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(54) Title: NEURONAL CELL-SPECIFIC PROMOTER

(57) Abstract: A polynucleotide is provided comprising a nucleotide sequence corresponding to the 5' promoter region of a glycine receptor alpha1 subunit gene or a derivative, variant or fragment of said sequence capable of conferring neuron-specific expression of a heterologous nucleotide sequence operably linked thereto.

NEURONAL CELL-SPECIFIC PROMOTERField of the invention

The present invention relates to a promoter which confers neuron-specific expression. The promoter may be used to direct expression of therapeutic gene products specifically in neurons, such as motor neurons.

Background to the invention

Traumatic spinal cord damage results in severe and lifelong disability and, in the worst instance, leads to irreversible paralysis below the site of injury. Reversing the permanence of paralysis remains one of the greatest challenges in medical science, as no treatment for the recovery of motor functions after spinal cord injury is presently available. Progress made in spinal cord research over the last decade has started to shed some light into the cellular and molecular mechanisms underlying neuronal survival and axonal regeneration. For instance, there is now good evidence that neurotrophic factors, which are proteins that promote the growth of axons and that contribute to neuronal maturation during the ontogenesis of the central nervous system (CNS), can assist the regeneration of the mature central nervous system. Indeed, some members of the neurotrophin family such as glial-cell-line-derived neurotrophic factor (GDNF) and neurotrophin-3 (NT-3) have been shown to stimulate axon collateral sprouting and prevent the axotomy-induced death of corticospinal neurons. More importantly, it has been recently demonstrated that these neurotrophins can stimulate the recovery of motor functions (Ramer et al., 2000, Nature 403: 312-316). However, systemic administration of GDNF or NT-3 is of limited value since it has been reported that the dose required to generate a therapeutic effect is above the toxicity threshold in humans.

The CNS is a soft structure that is guarded against possible mechanical damage by the skull and the vertebrae as well as by the meninges. Additionally, the CNS relies on the blood-brain barrier for its defence against the presence of foreign agents that could be present in the bloodstream. These physical and

chemical protective mechanisms hardly make the CNS amenable to clinical treatment. However, spinal cord motor neurons innervate the skeletal musculature with their peripherally projecting axons, and this offers an indirect route of administration of therapeutic agents. Gene therapy has taken advantage of this distinctive feature of motor neurons to deliver neurotrophic factors to the spinal cord. Indeed, it is possible to transduce motor neurons with neurotrophic factors by injecting vectors containing the genes that encode for these factors into the peripheral musculature. When injected in a muscle, the vectors carrying neurotrophic factor genes are taken up by the axon terminals that are present at the injection site and the therapeutic agents are subsequently transported, in a retrograde fashion, to the motor neurons from which these axons originate.

The delivery and expression of genetic material can also be achieved with viral systems such as adenoviral vectors and non-viral gene delivery systems such as cationic liposomal DNA preparations. Non-viral systems have a number of advantages over viral systems. For example, they can be produced rapidly and at low cost. Moreover, unlike viral constructs, plasmid constructs are not limited by the size of the transgene they carry. Over the last decade, successful *in vivo* and *in vitro* cationic lipid-mediated DNA transfection (i.e., lipofection) of nerve cells has been repeatedly reported.

A number of groups have demonstrated that injection of naked DNA, i.e., without any special delivery system, into the skeletal musculature results in the uptake and sustained expression of the transgene in the muscle cells. Relatively efficient gene transfer in the CNS has also been achieved by direct injections of naked DNA. In the CNS, the transduction obtained with unconjugated DNA has been reported to be stable, although lower than that produced with other gene vehicles such as viral vectors or DNA plasmids complexed with cationic lipid. Gene delivery by means of a non-viral vector has recently attracted considerable interest with the development of an effective therapeutic DNA vaccines approach (Huang et al., 1999, Neuron 24:

639-647; for a review see Gurunathan et al., 2000, Ann. Rev. Immunol. 18: 927-974).

The viral cytomegalovirus (CMV) promoter has been widely used to direct the
5 expression of different transgenes in the CNS. However, the use of vectors containing the CMV promoter also results in the expression of genes in non-neuronal elements such as astrocytes, macrophages, and endothelial cells. Alternatively, other promoters such as the rat neuron-specific enolase (NSE),
10 the human synapsin 1 (SYN), and the rat tubulin α 1 (Ta1) gene promoters have also been used to limit the expression of transgene to neuronal cells (e.g., Twyman and Jones, 1997, J. Mol. Neuroscience 8: 63-73; Kugler et al., 2000, Mol. Cell. Neuroscience 17: 78-96).

One of the great challenges of gene therapy is the transfer of therapeutic genes
15 into the precise region of the CNS where the injury resides. This issue is particularly critical for the treatment of paralysis resulting from traumatic spinal cord injury, as the delivery of neurotrophic factors under the control of a non-neuron specific promoter in the peripheral musculature can have unpredictable effects. Although it is clear that an ideal promoter should be
20 cell-specific, no successful attempt has been made previously to design a nucleotide vector that is under the control of a motor neuron specific promoter.

Summary of the invention

We have demonstrated that an approximately 5 kb fragment of a glycine
25 receptor alpha1 subunit (GlyR alpha1) gene promoter confers neuronal cell-specific expression on a heterologous nucleotide sequence to which it is operably linked.

Accordingly, the present invention provides a polynucleotide comprising a
30 nucleotide sequence corresponding to the 5' promoter region of a glycine receptor alpha1 subunit gene or a derivative, variant or fragment of said

sequence capable of conferring neuron-specific expression of a heterologous nucleotide sequence operably linked thereto.

The present invention also provides a polynucleotide comprising a nucleotide sequence as shown in SEQ ID No. 1, or a homologue, derivative, variant or fragment of said sequence capable of conferring neuron-specific expression of a heterologous nucleotide sequence operably linked thereto. Particularly preferred fragments are nucleotides 1 to 5397 and nucleotides 2200 to 5397 of SEQ ID No. 1, or the equivalent regions in other GlyRalpha1 promoter sequences.

Preferably said neuron-specific expression is specific to motor neurons and/or neurons which express glycine receptors, which include motor neurons and interneurons.

Typically the polynucleotides of the invention are operably linked to a heterologous nucleic acid of interest (NOI) such that the polynucleotide directs expression of the NOI in a neuron, preferably a motor neuron and/or a neuron which expresses a glycine receptor, which includes motor neurons and interneurons.

The NOI may encode a polypeptide of therapeutic use such as a polypeptide which is cytotoxic, a polypeptide capable of converting a precursor prodrug into a cytotoxic compound or a polypeptide selected from polypeptides involved in the regulation of cell division, enzymes involved in cellular metabolic pathways, neurotrophic factors, transcription factors and agents such as antibodies or polypeptides which block the action of inhibitory factors.

The present invention also provides a nucleic acid vector comprising a polynucleotide of the invention.

Polynucleotides and nucleic acid vectors of the invention may be combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition.

- 5 Polynucleotides, nucleic acid vectors and pharmaceutical compositions of the invention may be used to deliver therapeutic genes to mammalian neurons, preferably motor neurons and/or neurons that express a glycine receptor, which include motor neurons and interneurons. More particularly, polynucleotides, nucleic acid vectors and pharmaceutical compositions of the invention may be
- 10 used to treat patients having conditions characterised by disease of and/or injury to the central nervous system (CNS). However, a number of disorders that may originate in the CNS only present symptoms in the other tissues, including the peripheral nervous system (PNS) and non-neural tissue. Thus patients with other disorders, such as disorders associated with, but necessarily
- 15 of, the CNS may also be treated where delivery of therapeutic genes to the CNS is of benefit.

Accordingly, the present invention provides a polynucleotide, nucleic acid vector and pharmaceutical composition of the invention for use in a method of treatment of a human or animal.

Also provided is a method of treatment of a human or animal patient suffering from a disease of, or injury to, the central nervous system, or associated with the central nervous system which method comprises administering an effective amount of a polynucleotide, a nucleic acid vector or a pharmaceutical composition of the invention to the patient in need of such treatment.

In a preferred embodiment, the polynucleotide, nucleic acid vector, viral vector or pharmaceutical composition is administered to the human or animal by injection into the tongue muscle of the human or animal. More preferably, the nucleic acid vector, viral vector or pharmaceutical composition is administered by non-viral means.

We have taken advantage of the direct innervation of individual muscles by glycine receptor-expressing spinal cord motor neurons to develop a system that uses retrograde axonal transport for delivery of genes specifically to glycine receptor-expressing neurons. We have shown for the first time that nucleic acid constructs free of complexing agents, such as cationic polymers are successfully transported to the CNS, in particular motor neurons. This system can therefore be used to direct the expression of therapeutic gene products specifically to brainstem and spinal cord motor neurons. The use of naked DNA in pharmaceutical compositions is advantageous compared with complexed DNA since it is difficult to obtain complexes of DNA with delivery vehicles such as cationic polymers that have predictable properties, as is required for pharmaceutical compositions. Accordingly, the demonstration herein for the first time that naked DNA can be successfully delivered to neuronal cells by retrograde axonal transport will assist in the preparation of suitable pharmaceutical formulations for the delivery of nucleic acids to neuronal cells.

Accordingly, in a further aspect, the present invention provides a method of delivering a nucleic acid to a neuronal cell by non-viral means which method comprises administering said nucleic acid as a naked nucleic acid substantially free of complexing agents, to muscle tissue such that the nucleic acid is transported to said neuronal cell by retrograde axonal transport. Preferably the nucleic acid comprises a promoter of the invention operably linked to a heterologous sequence encoding a gene product of interest.

25

We have also developed a system for identifying new sequences that direct expression specifically in neuronal cells and further characterising sequences such as the GlyR alpha1 promoter disclosed herein.

30 Thus, in a further aspect, the present invention also provides a method for determining whether a candidate nucleotide sequence is capable of conferring neuron-specific expression of a nucleotide sequence of interest operably linked thereto which method comprises:

- (i) providing a non-viral nucleotide vector comprising said candidate nucleotide sequence operably linked to a nucleic acid of interest;

(ii) administering said vector to the brain or brain stem of a non-human animal; and

5 (iii) determining whether said nucleotide sequence of interest is expressed specifically in neurons in the brain or brain stem of said animal.

Preferably said neurons are motor neurons and/or glycine receptor-expressing neurons. Preferably said specificity is in glycine receptor-expressing neurons.

The present invention also provides nucleotide sequences obtained by the above method of the invention, which sequence are capable of conferring neuron-specific expression of a nucleic acid of interest operably linked thereto, particularly motor neuron-specific expression and/or expression specific to glycine receptor expressing cells.

Detailed description of the invention

Unless defined otherwise, all technical and scientific terms used herein have
the same meaning as commonly understood by one of ordinary skill in the art
(e.g. in cell culture, molecular genetics, nucleic acid chemistry, hybridization
techniques and biochemistry). Standard techniques are used for molecular,
genetic and biochemical methods (see generally, Sambrook *et al.*, Molecular
Cloning: A Laboratory Manual, 2nd ed. (1989) Cold Spring Harbor Laboratory
Press, Cold Spring Harbor, N.Y. and Ausubel *et al.*, Short Protocols in
Molecular Biology (1999) 4th Ed, John Wiley & Sons, Inc. - and the full version
entitled Current Protocols in Molecular Biology, which are incorporated herein
by reference) and chemical methods.

30 A. Polynucleotides

Polynucleotides of the invention may comprise DNA or RNA. They may be single-stranded or double-stranded. They may also be polynucleotides which

- include within them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of
- 5 the present invention, it is to be understood that the polynucleotides described herein may be modified by any method available in the art. Such modifications may be carried out, for example, to enhance the *in vivo* activity or life span of polynucleotides of the invention.
- 10 Polynucleotides of the invention are typically in an "isolated" form. This means that are not in their naturally occurring form i.e. polynucleotides of the invention do not include GlyRalpha1 promoters present in the genome from which they originate together with the coding sequences which they normally direct expression of. Thus, when a polynucleotide of the invention is present
- 15 in a host cell, it is typically heterologous to that cell.

Promoter sequences

- Polynucleotides of the invention comprises a sequence based on the 5' promoter region of a glycine receptor alpha1 (GlyRalpha1) subunit. Such a
- 20 sequence may correspond directly to the 5' promoter region of a naturally occurring GlyRalpha1 gene (such as the human GLRA1 gene or mouse *Gla1* gene) or it may be a variant or derivative thereof as described below. GlyRalpha1 genes have been identified in a number of organisms including humans and mice. Consequently, a GlyRalpha1 promoter according to the
- 25 present invention from may be obtained from the 5' promoter region of any GlyRalpha1 gene from any organism whose genome comprises such a gene. Typically the GlyRalpha1 promoter will be of mammalian origin, such as mouse, rat or primate, more preferably human.
- 30 In a preferred embodiment, the GlyRalpha1 promoter of the present invention comprises the nucleotide sequence shown as SEQ ID NO. 1, or a homologue, derivative, variant or fragment thereof.

The terms "variant", or "derivative" in relation to the GlyRalpha1 promoter nucleotide sequence of the present invention includes any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or 5 more) nucleotides from or to the sequence provided that the resultant nucleotide sequence is capable of conferring neuron-specific expression of a heterologous nucleotide sequence operably linked thereto, preferably motor neuron and/or glycine receptor expressing neurons. Preferably, such a variant, or derivative directs expression to at least 50% of the levels obtained with the polynucleotide 10 sequence of SEQ ID No. 1, preferably at least 75% or 90%. Nucleotide sequences where large numbers of changes have been made such that the sequence is no longer recognisable to a person skilled in the art a promoter sequence of the invention are not intended to be encompassed by the terms "variant" and "derivative". Accordingly where variants and derivatives are produced by 15 modifying existing sequences, typically less than 10 or 20% of the sequence is modified, preferably less than 5%. For example, it is preferred that fewer than 100, 50 or 25 nucleotides be altered in a sequence having 1000 nucleotides.

However, as discussed below, this limits typically apply only to those regions 20 containing sequences that bind transcriptional components and therefore are responsible for transcriptional regulation. A person skilled in the art will appreciate that regions of the promoter sequence shown in SEQ ID No.1 for example are irrelevant to the functioning of the promoter and may be modified as desired. Examples of such regions include the repetitive repeat regions described 25 in Tables 3 and 4.

With respect to homologous sequences preferably there is at least 75%, more 30 preferably at least 85%, more preferably at least 90% homology to the sequences shown in the SEQ ID NO. 1. More preferably there is at least 95%, more preferably at least 98%, homology.

Calculation of % homology may be carried out using, for example, computer software that generates an optimum alignment and then produces a homology

- score such as the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A.; Devereux *et al.*, 1984, Nucleic Acids Research 12:387). Examples of other software than can perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel *et al.*, 1999 *ibid* – Chapter 18),
- 5 FASTA (Atschul *et al.*, 1990, J. Mol. Biol., 403-410) and the GENEWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching (see Ausubel *et al.*, 1999 *ibid*, pages 7-58 to 7-60).

However, the preferred sequence comparison program is the GCG Wisconsin
10 Bestfit program described above using the program defaults. The default scoring
matrix has a match value of 10 for each identical nucleotide and -9 for each
mismatch. The default gap creation penalty is -50 and the default gap extension
penalty is -3 for each nucleotide.

15 Promoter sequences comprise particular motifs (promoter elements) which act as
binding sites for transcription factors that regulate transcription from the
promoter. The sequence of these sites is more important than flanking "spacer"
regions for the activity of the promoter. Consequently, modifications to the
promoter sequence should avoid changes to the sequence of transcription factor
20 binding motifs which would significantly affect the binding of the corresponding
transcription factor. However, it is often possible to replace particular promoter
elements from the promoter of one gene with the an equivalent element from
another gene, which binds the same transcription factor. Such motif "swaps" are
within the scope of the present invention.

25

It may also be desirable to modify GlyRalpha1 promoters of the present invention
to increase or decrease the levels of expression in neurons such as glycine
receptor expressing cells. This may, for example be achieved by increasing the
number of promoter elements to allow binding of additional transcriptional
30 activator proteins and/or by making substitutions in motifs, such as motifs that
bind general transcriptional components of the RNA polymerase II transcription
machinery, to provide a stronger consensus sequence. For example, the proximal
promoter elements just upstream from the transcriptional initiation site (i.e. up to

about 200 to 300 nucleotides 5' of the transcriptional initiation site, which is at nucleotide 5399 of SEQ ID No. 1) may be replaced with other promoter elements that function in a similar manner.

- 5 In addition, a consequence of the way in which promoters are built up from various promoter elements is that overall homology comparisons may be less useful than comparisons of particular regions containing sequence elements. It is therefore preferred that homologous sequences have higher homology in these regions whereas intervening regions may have much lower homology. In this
10 regard, Table 4 in the Examples indicates the location in SEQ ID No. 1 of various regions of repetitive DNA. These regions are unlikely to contain promoter elements that confer cell-type specificity. Consequently, when carrying out homology comparisons, these regions are likely to be of low importance. Equally,
15 when modifying GlyRalpha1 promoter sequences of the present invention, substitutions, deletions and insertions in these regions may not affect to any significant extent the cell-type specificity of the promoter sequences of the invention.

- 20 The present invention also encompasses nucleotide sequences that are capable of hybridising selectively to the sequences presented herein. The term "selectively hybridisable" means that the polynucleotide used as a probe is used under conditions where a target polynucleotide of the invention is found to hybridise to the probe at a level significantly above background. The background hybridisation may occur because of other polynucleotides present, for example, in
25 the cDNA or genomic DNA library being screening. In this event, background implies a level of signal generated by interaction between the probe and a non-specific DNA member of the library which is less than 10 fold, preferably less than 100 fold as intense as the specific interaction observed with the target DNA. The intensity of interaction may be measured, for example, by radiolabelling the
30 probe, e.g. with ^{32}P .

Hybridisation conditions are based on the melting temperature (T_m) of the nucleic acid binding complex, as taught in Berger and Kimmel (1987, Guide to

Molecular Cloning Techniques, Methods in Enzymology, Vol 152, Academic Press, San Diego CA), and confer a defined "stringency" as explained below.

Maximum stringency typically occurs at about Tm-5°C (5°C below the Tm of
5 the probe); high stringency at about 5°C to 10°C below Tm; intermediate
stringency at about 10°C to 20°C below Tm; and low stringency at about 20°C to
25°C below Tm. As will be understood by those of skill in the art, a maximum
10 stringency hybridisation can be used to identify or detect identical
polynucleotide sequences while an intermediate (or low) stringency
hybridisation can be used to identify or detect similar or related polynucleotide
sequences.

In a preferred aspect, the present invention covers nucleotide sequences that can
hybridise to the nucleotide sequence of the present invention under stringent
15 conditions (e.g. 65°C and 0.1xSSC {1xSSC = 0.15 M NaCl, 0.015 M Na₃Citrate
pH 7.0}).

In a preferred embodiment, the GlyRalpha1 promoter sequence of the invention
comprises less than 10000 nucleotides preferably fewer than 8000 or 6000
20 nucleotides. Smaller fragments are also within the scope of the invention, such
as fragments having less than 5000, 4000, 3000, 2000 or 1000 nucleotides,
provided that such fragments are capable of conferring neuron-specific
expression of a heterologous sequence operably linked thereto, preferably
25 motor neuron-specific expression and/or glycine receptor expressing neuron-
specific expression. Preferred fragments comprise at least 200, 300, 400, 500 or
700 nucleotides. Highly preferred fragments comprise nucleotides 1 to 5397
(sequence 5' of the transcriptional start site) or nucleotides 2200 to 5397 of SEQ
ID No. 1, or the equivalent regions in other GlyRalpha1 promoter sequences.
Other preferred fragments may lack nucleotides 4621 to 5720 of the sequence
30 shown as SEQ ID No. 1, or equivalent regions of homologues, variants and
derivatives thereof. Consequently a preferred fragment consists essentially of

nucleotides 1 to 4620 or 2200 to 4620 of SEQ ID No. 1, or the equivalent regions in other GlyRalpha1 promoter sequences.

- The minimal region required to confer neuron-specific expression may be
- 5 determined by progressively deleting regions of, for example, the sequence shown as SEQ ID No. 1 and testing for specificity of expression using the expression assay described below which involves injection of constructs into the brain or brain stem of non-human animals, such as mice.
- 10 In addition to the GlyRalpha1 promoter sequence of the present invention, the polynucleotide of the invention may comprise additional regulatory control sequences. For example, additional levels of transcriptional control may be used to ensure that expression directed by the promoter sequence of the invention is confined to certain cells under certain conditions. Thus, for
- 15 example, additional enhancers may be operably linked to the GlyRalpha1 promoter sequence of the invention, either downstream, upstream or both.
- The additional regulatory sequence may be a sequence found in eukaryotic genes. For example, it may be a sequence derived from the genome of a cell in
- 20 which expression of directed by the promoter of the invention is to occur. In one embodiment the additional regulatory sequence is not a sequence that is naturally found operably linked to the promoter sequence of the present invention.
- 25 In most instances, these additional regulatory sequences, such as enhancers may be isolated as convenient restriction digestion fragments suitable for cloning in a selected vector. Alternatively, regulatory sequences may be isolated using the polymerase chain reaction. Cloning of the amplified fragments may be facilitated by incorporating restriction sites at the 5' end of
- 30 the primers. Regulatory sequences may also be synthesised using, for example, solid-phase technology.

It may also be desirable to include regulatory elements that are inducible, for example such that expression can be regulated by administration of exogenous substances. In this way, levels of expression directed by the promoter can be regulated during the life-time of the cell. Inducible means that the levels of expression obtained using the promoter can be regulated. For example, in a polynucleotide of the invention may comprises regulatory sequences responsive to the tet repressor/VP16 transcriptional activator fusion protein. Thus in this example, expression directed by the promoter of the invention would depend on the presence or absence of tetracycline.

10

An advantageous feature of the promoter sequences of the present invention is that they are neuron cell-specific. In particular, they are specific for cells that express strychnine sensitive glycine receptors comprising an alpha subunit. The term "cell specific" means a regulatory control sequence which is not necessarily restricted in activity to a single cell type but which nevertheless shows selectivity in that it is active in one group of cells and less active or silent in another group. However, it may be preferred that promoters of the invention show strict cell-specificity in that they are only active at detectable levels in neuronal cells, such as cells that express glycine receptors.

15

Preferred neuronal cells in which promoters of the present invention show specificity of expression are cells that express strychnine-sensitive glycine receptors comprising alpha subunits. Glycine receptors (GlyRs) are almost exclusively found on the cell surface of motor neurones located in the brainstem as well as throughout the full length of the spinal cord.

Nucleic acids of Interest (NOI)

Polynucleotides comprising a promoter of the invention are typically operably linked to an NOI, usually a heterologous gene. The term "heterologous gene" encompasses any gene. The heterologous gene may be any allelic variant of a wild-type gene, or it may be a mutant gene. The term "gene" is intended to cover nucleic acid sequences which are capable of being at least transcribed.

Thus, sequences encoding mRNA, tRNA and rRNA are included within this definition. The sequences may be in the sense or antisense orientation with respect to the promoter. Antisense constructs can be used to inhibit the expression of a gene in a cell according to well-known techniques. Nucleic acids may be, for example, ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or analogues thereof. Sequences encoding mRNA will optionally include some or all of 5' and/or 3' transcribed but untranslated flanking sequences naturally, or otherwise, associated with the translated coding sequence. It may optionally further include the associated transcriptional control sequences normally associated with the transcribed sequences, for example transcriptional stop signals, polyadenylation sites and downstream enhancer elements. Nucleic acids may comprise cDNA or genomic DNA (which may contain introns). However, it is generally preferred to use cDNA because it is expressed more efficiently since intron removal is not required.

15

The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequence.

In accordance with the present invention, suitable NOI sequences include those that are of therapeutic and/or diagnostic application such as, but are not limited to: sequences encoding cytokines, chemokines, hormones, antibodies, engineered immunoglobulin-like molecules, a single chain antibody, fusion proteins, enzymes, immune co-stimulatory molecules, immunomodulatory molecules, anti-sense RNA, a transdominant negative mutant of a target protein, a toxin, a conditional toxin, an antigen, a tumour suppressor protein and growth factors, membrane proteins, anti-viral proteins and ribozymes, and derivatives thereof (such as with an associated reporter group).

NOI(s) may be used which encode polypeptides, antibodies, antisense transcripts or ribozymes which interfere with expression of cellular genes, such as inhibitory proteins that inhibit the action of neurotrophins.

- 5 Suitable NOIs for use in the present invention may also include NOIs that may be used in the treatment or prophylaxis of cancer include NOIs encoding proteins which: destroy the target cell (for example a ribosomal toxin), act as: tumour suppressors (such as wild-type p53); activators of anti-tumour immune mechanisms (such as cytokines, co-stimulatory molecules and immunoglobulins); or which provide enhanced drug sensitivity (such as pro-drug activation enzymes); indirectly stimulate destruction of target cell by natural effector cells (for example, strong antigen to stimulate the immune system or convert a precursor substance to a toxic substance which destroys the target cell (for example a prodrug activating enzyme). Encoded proteins could
- 10 also destroy bystander tumour cells (for example with secreted antitumour antibody-ribosomal toxin fusion protein), indirectly stimulated destruction of bystander tumour cells (for example cytokines to stimulate the immune system or procoagulant proteins causing local vascular occlusion) or convert a precursor substance to a toxic substance which destroys bystander tumour cells
- 15 (e.g. an enzyme which activates a prodrug to a diffusible drug).
- 20

Instead of, or as well as, being selectively expressed in target tissues, the NOI or NOIs may encode a pro-drug activation enzyme or enzymes which have no significant effect or no deleterious effect until the individual is treated with one or more pro-drugs upon which the enzyme or enzymes act. In the presence of the active NOI, treatment of an individual with the appropriate pro-drug leads to enhanced reduction in tumour growth or survival.

In each case, a suitable pro-drug is used in the treatment of the patient in combination with the appropriate pro-drug activating enzyme. An appropriate pro-drug is administered in conjunction with the NOI. Examples of pro-drugs include: etoposide phosphate (with alkaline phosphatase); 5-fluorocytosine (with cytosine deaminase); doxorubicin-N-p-hydroxyphenoxyacetamide (with

penicillin-V-amidase); para-N-bis(2-chloroethyl) aminobenzoyl glutamate (with carboxypeptidase G2); cephalosporin nitrogen mustard carbamates (with β -lactamase); SR4233 (with P450 Reducase); ganciclovir (with HSV thymidine kinase); mustard pro-drugs with nitroreductase and cyclophosphamide (with 5 P450).

- Examples of suitable pro-drug activation enzymes for use in the invention include a thymidine phosphorylase which activates the 5-fluoro-uracil pro-drugs capcetabine and furtulon; thymidine kinase from herpes simplex virus 10 which activates ganciclovir; a cytochrome P450 which activates a pro-drug such as cyclophosphamide to a DNA damaging agent; and cytosine deaminase which activates 5-fluorocytosine. Preferably, an enzyme of human origin is used.
- 15 The expression products encoded by the NOIs may be proteins which are secreted from the cell. Alternatively the NOI expression products are not secreted and are active within the cell.
- Particularly preferred NOIs encode neurotrophic factors, such as nerve growth 20 factor (NGF), β -NGF, ciliary neurotrophic factor (CNTF) brain-derived neurotrophic factor (BNTF), glial cell line derived neurotrophic factor (GDNF) and neurotrophins such as NT-3, NT-4, NT-5. For example, it has been shown that delivery of the NT-3 gene has led to an improvement in motor functions in a mouse model (Haase et al., 1997, Nat. Med 3: 380-381).
25 Preferred NOIs may also encode transcription factor Brn-3a (or an N-terminal fragment thereof) which has been shown to protect neuronal cells from apoptosis (WO99/05202). Brn-3a activates specifically expression of the Bcl-2 gene in neuronal cells: this activation is mediated via a Brn-3a response 30 element in the 5' regulatory region of the Bcl-2 gene.

The NOI may encode antibodies such as IN-1 that neutralise the action of Nogo-A, one of the major inhibitory proteins expression in CNS myelin (Chen

et al., 2000, *Nature* 403: 434-439), or antibodies or other proteins or reagents that inhibit classes of molecules such as the semaphorins, ephrins, etc which are known to have inhibitory or repulse effects on axonal regrowth. The therapeutic use of antibodies to Nogo-A is described in Merkler et al., 2001,
5 *J. Neuroscience* 21: 3665-3673.

The use of combinations of the above NOIs is also envisaged.

NOIs may also include marker genes (for example encoding beta-galactosidase
10 or green fluorescent protein) or genes whose products regulate the expression of other genes (for example, transcriptional regulatory factors including the tet repressor/VP16 transcriptional activator fusion protein described above). In addition, NOIs may comprise sequences coding fusion protein partners in frame with a sequence encoding a protein of interest. Examples of fusion
15 protein partners include the DNA binding or transcriptional activation domain of GAL4, a 6xHis tag and beta-galactosidase. It may also be desirable to add targeting sequences to target proteins encoding by NOIs to particular cell compartments or to secretory pathways. Such targeting sequences have been extensively characterised in the art.

20

C. Nucleic Acid Vectors

Polynucleotides of the invention can be incorporated into a recombinant vector, typically a replicable vector. The vector may be used to replicate the nucleic acid in a compatible host cell. Thus in a further embodiment, the invention
25 provides a method of making polynucleotides of the invention by introducing a polynucleotide of the invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector. The vector may be recovered from the host cell. Suitable host cells include bacteria such as *E. coli*, yeast, mammalian
30 cell lines and other eukaryotic cell lines, for example insect Sf9 cells.

A vector comprising a polynucleotide of the invention which is operably linked to an NOI can be considered to be an expression vector since under suitable

- conditions, the NOI will be expressed under the control of the promoter construct of the present invention. However, it is not necessary for a vector of the invention to comprise an NOI. Nonetheless it is possible to introduce an NOI into the vector at a later stage. Thus a vector of the invention which lacks
- 5 an NOI can be considered to be a cloning vector. Preferably, a cloning vector of the invention comprises a multiple cloning site downstream of the GlyRalpha1 promoter sequences to enable an NOI to be cloned into the vector when required whereby it is then operably linked to the GlyRalpha1 promoter sequences.
- 10 The vectors may be for example, plasmids, chromosomes, artificial chromosomes or virus vectors. The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene in the case of a bacterial plasmid or a neomycin resistance gene for a mammalian vector.
- 15 Vectors may be used, for example, to transfect, transform or transduce a host cell either *in vitro* or *in vivo*.

D. Delivery of nucleic acid vectors to neuronal cells

- 20 Nucleic acid vectors of the present invention may be delivered to neuronal cells, such as motor neurons and/or glycine receptor-expressing neurons by viral or non-viral means. However, it is preferred to use non-viral means.

Non-viral delivery systems include but are not limited to DNA transfection methods. Here, transfection includes a process using a non-viral vector to

25 deliver a gene to a target mammalian cell. Typical transfection methods include electroporation, DNA biolistics, lipid-mediated transfection, compacted DNA-mediated transfection, liposomes, immunoliposomes, lipofectin, cationic agent-mediated, cationic facial amphiphiles (CFAs), multivalent cations such as spermine, cationic lipids or polylysine, 1, 2,-bis (oleoyloxy)-3-

30 (trimethylammonio) propane (DOTAP)-cholesterol complexes and combinations thereof. Alternatively, polynucleotides/nucleic acid vectors of the invention may be naked polynucleotide constructs in the sense that they

may be free from any delivery vehicle, which would act to facilitate entry into the cell (e.g. viral sequences) and/or to promote transfection (e.g., liposomes, polybrene, divalent cations).

- 5 It is preferred to use such naked polynucleotide constructs to avoid complications and side effects that may arise from the use of delivery vehicles. Further, the use of naked DNA in pharmaceutical compositions is advantageous compared with complexed DNA since it is difficult to obtain complexes of DNA with delivery vehicles such as cationic polymers that have predictable properties, as is required for pharmaceutical compositions.
- 10

Preferably, the sequences used in the method of the invention do not integrate into the genome of the host cell, but rather remain in the cell as episomal elements.

15

- Viral delivery systems include but are not limited to an adenovirus vector, an adeno-associated viral (AAV) vector, an alphavirus vector, a herpes viral vector, a retroviral vector, such as a lentiviral vector and combination vectors such as an adenolenti viral vector. In the case of viral vectors, gene delivery is typically mediated by viral infection of a target cell.
- 20

- Generally, target cells will be present in a living multicellular organism. Administration may be by direct introduction into the site to be treated, for example by injection into the brain, brain stem or spinal cord. Alternatively, 25 administration may be by indirect means such as by injection into the tongue or other muscle. The polynucleotides may then reach the central nervous system by axonal retrograde transport to neuronal cell bodies in the brain stem.

E. Therapeutic uses and administration

- 30 The polynucleotides, vectors and compositions of the present invention may be used to treat diseases of the nervous system which affect neurons such as motor neurons. For example, the polynucleotide, nucleic acid vectors of the invention

may be used to deliver therapeutic genes to a human or animal in need of treatment.

Diseases which may be treated, prevented or alleviated include diseases of the

5 central nervous system such as neurodegenerative diseases and damage to nervous tissue as a result of injury/trauma (including strokes and spinal cord injuries).

However, a number of disorders that may originate in the CNS only present

10 symptoms in the other tissues, including the peripheral nervous system (PNS) and non-neural tissue. Thus patients with other disorders, such as disorders associated with, but necessarily of, the CNS may also be treated where delivery of therapeutic genes to the CNS is of benefit.

15 Specific diseases or conditions that may be treated include inflammatory components of strokes, post-polio syndrome, acute neuropathy, subacute neuropathy, chronic neuropathy, pseudo-tumour cerebri, Huntington's disease, amyotrophic lateral sclerosis, inflammatory components of CNS compression or CNS trauma or infections of the CNS, conditions or disorders of the central and

20 peripheral nervous systems. In particular, neurodegenerative diseases include motor neuron disease, several inherited diseases, such as familial dysautonomia and infantile spinal muscular atrophy, hyper-ekplexia, and late onset neurodegenerative diseases such as Parkinson's and Alzheimer's diseases.

25 The polynucleotides, vectors and compositions of the present invention which express therapeutic genes may be used to stimulate the growth of axons, neuronal maturation and/or neuronal regeneration.

30 The polynucleotides of the invention may be administered directly to a patient in need of therapy as a naked nucleic acid construct. Uptake of naked nucleic acid constructs by mammalian cells is enhanced by several known transfection techniques for example those including the use of transfection agents. Example of these agents include cationic agents (for example calcium phosphate and

DEAE-dextran) and lipofectants (for example lipofectam™ and transfectam™). Thus nucleic acid constructs may be mixed with the transfection agent to produce a composition. However, it is preferred that compositions of the invention lack nucleic acid complexing agents such as cationic lipids.

5

Preferably the naked nucleic acid construct or vector is combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition may be formulated for
10 parenteral, intramuscular, intravenous, subcutaneous, intraocular or transdermal administration. The pharmaceutical composition may be for human or animal usage.

The pharmaceutical composition is administered in such a way that the
15 polynucleotide of the invention can be incorporated into cells at an appropriate area. For example, when the target of gene therapy is the central or peripheral nervous system and the polynucleotide of the invention is to be delivered by a herpes simplex virus vector, the composition could be administered in an area where synaptic terminals are located so that the virus can be taken up into the
20 terminals and transported in a retrograde manner up the axon into the axonal cell bodies via retrograde axonal transport. The pharmaceutical composition is typically administered to the brain by stereotaxic inoculation. When the pharmaceutical composition is administered to the eye, sub-retinal injection is typically the technique used.

25

In a preferred embodiment, polynucleotides are administered non-virally via retrograde axonal transport i.e. intramuscular injection of non-viral DNA so that the DNA is taken up and transported to motor neurons by retrograde axonal transport. One technique involves injection into the tongue muscle as
30 described in Wang et al., 2001, Mol. Ther 3: 658-664. However, Wang et al. use DNA complexed/conjugated to cationic polymers. By contrast, we have shown that naked DNA can be injected into the tongue muscle and transported to motor neurons by retrograde axonal transport, without the requirement for

complexing agents that may have undesirable physiological side-effects. It is therefore preferred to administer nucleic acids as naked DNA, typically formulated as a composition with a pharmaceutically acceptable carrier or diluent.

5

- When the polynucleotide of the invention is delivered to cells by a viral vector, the amount of virus administered is in the range of from 10^3 to 10^9 pfu, preferably from 10^5 to 10^7 pfu. When injected, typically 1-10 μ l of virus in a pharmaceutically acceptable suitable carrier or diluent is administered. When 10 the polynucleotide/vector is administered as a naked nucleic acid, the amount of nucleic acid administered is typically in the range of from 1 μ g to 10 mg, preferably from 100 μ g to 1 mg.

- Where the polypeptide of the invention is under the control of an inducible 15 promoter, it may only be necessary to induce gene expression for the duration of the treatment. Once the condition has been treated, the inducer is removed and expression of the NOI ceases. This will clearly have clinical advantages. Such a system may, for example, involve administering the antibiotic tetracycline, to activate gene expression via its effect on the tet repressor/VP 16 20 fusion protein.

- The use of neuron-specific promoters of the invention will be of assistance in the treatment of diseases of the nervous system. For example, several neurological disorders are due to aberrant expression of particular gene 25 products in only a small subset of cells. It will be advantageous to be able express therapeutic genes in only the relevant affected cell types, especially where such genes are toxic when expressed in other cell types. Examples of neuronal sub-types which may be targeted specifically include motor neurons.
- 30 The routes of administration and dosages described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and dosage for any particular patient and condition.

The efficacy of any given NOI in treating conditions relating to the nervous system may typically be tested using an animal model. For example, it has been shown that delivery of the NT-3 gene has led to an improvement in motor functions in a mouse model (Haase et al., 1997, Nat. Med 3: 380-381). Mouse models of neuronal function are considered in the art to be predictive in assessing the applicability of these treatments to humans.

F. Assays for neuron-cell specific expression

The present invention also provides methods for assaying promoter constructs such as fragments, derivatives and variants of GlyRalpa1 promoters for activity as neuron-cell specific regulatory control sequences.

These methods comprise providing suitable nucleic acid constructs which comprise a candidate nucleotide sequence whose activity it is desired to test operably linked to a heterologous nucleic acid sequence of interest. The heterologous NOI is selected such that its expression in neuronal cells is detectable. It is especially preferred to use NOIs whose expression can be detected histologically so that the particular cells in which it is expressed can be readily seen in sections of tissue of the CNS. Examples of suitable NOIs include beta-galactosidase and green fluorescent protein or variants thereof (e.g. blue fluorescent protein).

The nucleic acid constructs are administered directly to the brain or brain stem of a non-human animal such as a mouse or a rat using suitable means such as direct injection, for example direct injection into the amygdala (see the Examples).

The animal is then typically allowed to recover and is kept for a period of time, such as 4 to 6 days, to allow retrograde transport of the polynucleotide constructs and expression of the gene product.

Animals are then sacrificed and brain and/or brain stem tissue removed for subsequent analysis to determine whether expression of the NOI is specific to neuronal cells and in particular subsets of neuronal cells such as motor neurons and/or glycine receptor expressing cells. This may be achieved, for 5 example, by taking thin sections of brain tissue and visualising products of expression of the NOI by immunohistochemistry and microscopy. Typically, expression specific to glycine receptor-expressing neurons is indicated when expression is seen in the glycine receptor-expressing parabrachial nucleus but not the glycine receptor-lacking insular cortex.

10

Promoter sequences identified by the above method may be used in polynucleotides constructs of the present invention.

It should be appreciated that features from various sections, aspects and 15 embodiments of the invention as described above are generally equally applicable to other sections, aspects and embodiments *mutatis mutandis*.

The invention will now be further described by way of Examples, which are meant to serve to assist one of ordinary skill in the art in carrying out the 20 invention and are not intended in any way to limit the scope of the invention. The Examples refer to the Figures. In the Figures:

Figure 1 EGFP-positive neurons in the hypoglossal nucleus after an injection, in the tongue, of the fusion construct under the control 25 of the 5kb hGlyR α 1 promoter.

Figure 2 Predicted results from an injection, in the central nucleus of the amygdala (Ce), of the IRES construct under the control of the NSE (upper part) and of the fusion construct under the 5kb hGlyR α 1 promoters (lower part). The NSE promoter was hypothesised to give rise to EGFP-positive neurons in both the insular cortex (IC) 30 and the parabrachial nucleus (PB). The 5kb hGlyR α 1 promoter

was hypothesised to give rise to EGFP-positive neurons in the PB only.

5 Figure 3 EGFP-positive neurons in both the insular cortex (IC) (left) and the parabrachial nucleus (PB) (right) after an injection, in the central nucleus of the amygdala (Ce) of the IRES construct under the control of the NSE promoter.

10 Figure 4 Absence of EGFP-positive neurons in both the insular cortex (IC) (left), but presence of EGFP-positive neurons in the parabrachial nucleus (PB) (right) after an injection, in the central nucleus of the amygdala (Ce) of the fusion construct under the control of the the 5kb hGlyR α 1 promoter.

15 EXAMPLES

Construct design

Two types of constructs were generated to evaluate the function and specificity of the glycine receptor 5' promoter. The first type of construct used the pIRES-20 EGFP vector (Clontech Laboratories Inc, CA, USA) that allows the bicistronic expression of the human glycine receptor alpha1 subunit (hGlyR α 1) transgene and the enhanced green fluorescent protein (EGFP) reporter gene. With this type of construct the EGFP remains in the cytoplasm of the transfected cells whilst the hGlyR α 1 is localised to the cell membrane. The 25 second type of construct, which is based on the pcDNA3.1 (-) plasmid (Invitrogen Corp., NL) expresses the EGFP and the hGlyR α 1 as a fusion protein. The fusion protein is expected to behave in the same manner as the native membrane bound receptor. The hGlyR α 1 cDNA was subcloned from pCishGlyR α 1 (in our laboratory). The 1745 bp insert contains the entire 30 open reading frame, 287 bp of 5' UTR and 115 bp 3' UTR.

IRES constructs

In the pIRES-EGFP vector, the *Xba*I site at 2909bp was deleted and replaced with a *Not*I site, by insertion of a double stranded linker (GlyRTgXho2). The *Nru*I to *Eco*RV fragment containing the CMV promoter was excised and the 5 blunt ends ligated (pIX-CMV). A pIRES-EGFP based vector without the *Xba*I deletion, *Not*I replacement, and without the CMV promoter removed was also used (pIRES-EGFP-CMV).

The rat neuron-specific enolase promoter was used as a control and 1.8kb of 10 promoter was excised from pNSElacZ (gifted by Ora Bernard) with *Eco*R1 and *Hind*III restriction digest. The 1745 bp of hGlyRalpha1 coding sequence was amplified with the addition of an *Eco*R1 site and a *Hind*III site, or a *Kpn*I and a *Not*I site (Table 1).

15 Human GlyRalpha1 Gene Promoter

The structure and sequence of the hGlyRalpha1 promoter is shown in SEQ ID No. 1. The coding region and 5' UTR of hGlyRalpha1 is shown in SEQ ID No. 2. A region 1097 bases of 5' sequence upstream from the translational start site defined by the sequence ATG, excluding the 5' UTR already contained in 20 pCis hGlyRalpha1, was amplified using primers 5' GlyRP2FEco and 3' GlyRP1RevHind with the addition of restriction sites for cloning (Table 1). Human DNA, extracted from blood, was used as a template in the PCR. The hGlyRalpha1 cDNA was amplified from pCis hGlyRalpha1 with 5' GlyRP5Hind and 3' GlyRP6Eco primers containing restriction sites for cloning. The resulting 25 PCR fragment was digested with *Eco*RI and *Hind*III and ligated either with the NSE *Eco*R1-*Hind*III fragment or the 793 bp GlyR promoter fragment. Ligation products were separated on a 0.8% 1x TAE agarose gel. Products of the correct size were excised, purified and cloned into the *Eco*R1 site in PIX-CMV.

30 The human glycine receptor alpha1 (hGlyRalpha1) subunit gene is located on chromosome 5. From cosmid 77H1, donated to us by Rita Shiang, we determined the DNA sequence of 4620 bp upstream of the published hGlyRalpha1 promoter sequence. Sequence was confirmed on the reverse

strand. In total, 5723 bp upstream of the translational start site was sequenced. This sequence was subjected to a BLAST search to look for expressed sequence tags (ESTs) from other genes. None were found, indicating that the sequence contained only promoter sequences.

5

- A restriction map was constructed and a 3205 bp 5' promoter sequence was PCR amplified with the addition of a *NotI* site at the 5' end and a *KpnI* site at the 3' end. The hGlyRalpha1 cDNA was amplified from pCis hGlyRalpha1 with primers containing *NotI* and *KpnI* restriction sites for cloning. The resulting 10 PCR fragments were digested with *NotI* and *KpnI* and ligated together. Ligation products were separated on a 0.8% 1x TAE agarose gel. Products of the correct size were excised, purified and cloned into pIRES-EGFP-CMV).

Table 1

15

Primer name	Size	Sequence 5'-3'
GlyRTgXho2	14	TCGATGCGGCCGCA (SEQ ID No. 3)
GlyRF2FEco	25	GGGAATTCCGCCAGATCTCGTCCAG (SEQ ID No. 4)
GlyRP1RevHind	30	CCAGCGTGTCAAGCTTCTCCCTGC GGCGCT (SEQ ID No. 5)
GlyRP8NotF	28	TTTTGGCGGCCGCTATATCCCCAGTGC (SEQ ID No. 6)
GlyRP7NotF	28	GACATGGCGGCCGCCAGCACAGTGTAG (SEQ ID No. 7)
GlyRP9KpnF	26	CAGACACGCTGGTACCTAACAAACAG (SEQ ID No. 8)
GlyRP10KpnR	26	CTGTTGTTAGGTACCAGCGTGTCTG (SEQ ID No. 9)
GlyRP11NotR	24	GCTTGGCGGCCGCTCGACTCTAG (SEQ ID No. 10)

GlyRP5HindIII	21	ATCAAGCTTGACACGCTGGAG (SEQ ID No. 11)
GlyRP6EcoRI	21	TAGAATTCGCTGCAGGTCGAC (SEQ ID No. 12)

Fusion constructs

Site-directed mutagenesis using primers SacII F and SacII R were used to introduce a restriction site into hGlyRalpha1 (in pCis) at nt 381, to enable 5 insertion of the EGFP gene (Table 2). The hGlyRalpha1 subunit gene was then amplified with primers GlyRP6EcoRI and GlyRP7EcoRI. The PCR product was digested with EcoRI and cloned into the EcoRI site of pcDNA3.1 (-). The EGFP gene was amplified by PCR from pIRES-EGFP with primers EGFPSSacIIF and EGFPSSacIIR, and the 717 bp product digested with SacII and subcloned into 10 hGlyRalpha1. The CMV promoter was subsequently removed by digesting with *Mlu*I and *Xba*I and treating the overhangs with Mung bean nuclease. Fragments of the hGlyRalpha1 promoter differing in size were then cloned into the *Not*I site in the MCS. Three fragments of the hGlyRalpha1 promoter were amplified by PCR from the cosmid provided by Rita Shiang. The primers used were; 15 GlyRP2NotF and GlyRP1rNot for the 786 bp product, GlyRP8NotF and GlyRP1rNot for the 3191 kb product, GlyRP7NotF and GlyRP1rNot for the 5412 bp product (Table 1, Table 2). The PCR products were separated on a 0.8% 1x TAE agarose gel. Products of the correct size were excised, purified and cloned into the modified pcDNA3.1 (-) containing hGlyRalpha1 fused with EGFP.

20

Table 2

Primer name	Size	Sequence 5'-3'
GlyRP1rNot	30	CCAGCGTGTGCGGCCGCTCCCTGCGGCGCT (SEQ ID No. 13)
GlyRP2Fnot	25	GGGGATGCGGCCGCATCTCGTCCAG (SEQ ID No. 14)

GlyRP1Kpn	29	CCAGCGTGTCTGTTGGTACCCCTGC GGCGC (SEQ ID No. 15)
EGFPSacIIR (#6)	26	AGGCCGTGGTGTACAGCTCGTCCATG (SEQ ID No. 16)
EGFPSacIIF (#5)	24	AGGCCGCGGTGAGCAAGGGCGAGG (SEQ ID No. 17)
SacII F	28	CTGCTCGCTCCGCGGCCAAGCCTATGTC (SEQ ID No. 18)
SacIIR	28	GACATAGGCTTGGCCGGAGCGAGCAG (SEQ ID No. 19)
GlyRP6EcoRI	21	TAGAATT CGCTGCAGGTCGAC (SEQ ID No. 20)
GlyRP7EcoRI	19	ATCGAATT CGACACGCTGG (SEQ ID No. 21)

Example 1 - Retrograde transport and expression of the GlyR gene constructs in the CNS

We initially selected the tongue musculature as the injection site based on the anatomical evidence that neurons in the brainstem hypoglossal nucleus, which is the 12th cranial motor nucleus, innervate the tongue via the 12th cranial nerve. In addition, the muscles of the tongue are easily accessible, so tongue injections provide a simple and relatively non invasive way to evaluate whether injections of our different gene constructs in the peripheral musculature can result in the transport and expression of the reporter gene in the CNS.

Methods

Injections of DNA. C57BL/6 male mice, weighing 17-25 g at the time of surgery, were used in this experiment. Surgery was performed under aseptic conditions. The mice were deeply anaesthetised with a mixture of ketamine (100 mg/ml) and xylazine (20 mg/ml) (80 mg/kg ip) and placed on a heating pad. The animal's tongue was gently pulled out with a pair of forceps and injected with 8 µl of DNA (approximately 5 µg in distilled water) through a 0.5 cc

insulin syringe (Becton Dickenson, Singapore) at a single point in the midline of the tongue.

The mice were monitored until fully recovered from anaesthesia and then
5 returned to the animal room where they were kept for a minimum of 6 days to allow for the retrograde transport, along the 12th cranial nerve, and the expression of the reporter gene in the hypoglossal nucleus.

Histology. One week after the surgery, the mice were anaesthetised with a
10 lethal dose of sodium pentobarbital and perfused through the heart with 0.1 M phosphate buffer saline followed by a solution of paraformaldehyde (4% in 0.1 M phosphate buffer saline). The brainstems were dissected out, post-fixed, embedded in paraffin, and cut into 10 µm-thick coronal sections. The sections were floated in a 42° C water bath and then mounted on microscope slides.
15 The slides were heated at 60° C and subsequently immersed in a histological clearing agent (for 15 min, followed by quick rinses in graded ethanols).

Immunohistochemistry The tissue was rehydrated in 0.1 phosphate buffer saline (PBS), placed in boiling citrate buffer (0.1 M citric acid and 0.1 M trisodium citrate, pH 6.0) for 3 min to retrieve the antigen, washed again in 0.1 PBS, and incubated in a solution of bovine serum albumin and normal goat serum (1% and 10%, respectively in 0.1 PBS) for 20 min. The tissue was then incubated with a rabbit polyclonal antibody raised against the marker protein EGFP (anti-GFP 290, Abcam, Cambridge, UK) (dilution, 1:100 in 0.5% bovine serum albumin and 2% normal goat serum in 0.1 M PBS) for 30 min. After incubation in the primary antibody, the tissue was washed with 0.1 PBS and incubated in the dark for 30 min in Alexa Fluor 488 goat anti-rabbit secondary antiserum (Molecular Probe, Eugene, OR, USA) (dilution, 1:100 in 0.5% bovine serum albumin and 2% normal goat serum in 0.1 M PBS). The tissue was 25 subsequently washed in PBS, coverslipped with fluorescent mounting medium (DAKO, CA, USA), and kept in light-tight boxes.
30

Microscopic analysis. The brain tissue was analysed to visualise the EGFP-labelled neurons with a laser scanner confocal microscopr (Leica TCS SP) equipped with a krypton/argon laser. The images were acquired with exitation at 488nm and they were exported into Photoshop for presentation.

5

Results

Figure 1 shows some EGFP-positive neurons in the hypoglossal nucleus resulting from the injection of the EGFP-GlyR fusion construct in the tongue musculature of a C57BL/6 mice. As seen in this figure, the protein marker
10 EGFP is abundant in these neurons, confirming that the injection of the EGFP-GlyR construct in the tongue musculature has resulted in the retrograde transport, along the twelfth cranial nerve, and the expression of the transgene in hypoglossal neurons (see Figure 1). The results of this experiment have therefore established that, when injected in the peripheral musculature, the
15 non-viral delivery system can transfer genes successfully in the CNS and that these constructs can express an NOI in the target neurons.

Both circular plasmid and linearised plasmid were injected, but there was no noticeable difference in EGFP expression.

20

Example 2 - The specificity of expression of the GlyR gene promoter

To assess the specificity of expression of the GlyR gene promoter, its pattern of expression was compared to that of the rat neuron-specific enolase (NSE) promoter that is known to direct neuron-specific expression of the transgene.
25 GlyRalpha1 constructs under the control of these two promoters were injected in the central nucleus of the amygdala (Ce) in C57BL/6 mice. The Ce was selected as the brain structure of choice because of its distinctive connectivity with the rest of the CNS. Figure 2 is a diagrammatic representation of the connectivity of the Ce. The Ce receives a projection from both the parabrachial
30 nucleus (PB), a brainstem area in which GlyR are highly expressed, and from the insular cortex (IC) where neurons do not express GlyR. It was hypothesised that an injection of the gene construct that is under the control of the NSE

promoter in the Ce would result in the retrograde transport and expression of the transgene in both the IC and the PB. On the other hand, it was predicted that the injection of the gene construct that is under the control of the GlyR gene promoter would be likewise transported to both the IC and the PB, but 5 would only be expressed in the PB (see Figure 2).

Methods

- Injections of DNA C57BL/6 male mice, weighing 17-25 g at the time of surgery, were used in this experiment. Surgery was performed under aseptic 10 conditions. The mice were deeply anaesthetised with a mixture of ketamine (100 mg/ml) and xylazine (20 mg/ml) (80 mg/kg ip) and placed on a heating pad, in a stereotaxic apparatus. The head was positioned so that lambda and bregma points were horizontally aligned. An incision was made with a scalpel on the mouse's skin to expose the skull and a small hole was drilled in the skull over 15 the region of interest. The central nucleus of the amygdala was infused over 2 min with 2 µl of DNA (approximately 5 µg in distilled water) through a 33-gauge cannula connected to a 1cc syringe (Becton Dickenson, Singapore) driven by an infusion pump (KdScientific, New Hope, PA, USA). The stereotaxic coordinates used for the injections were AP, -1.1; ML, 2.4; DV, -4.0.
- 20 After the completion of the injections, the cannula was left in place inside the mouse's brain for an additional 5 min to minimise diffusion, after which the wound was cleaned and the skin incision sutured. Topical analgesic ointment (lignocaine and prilocaine, 25 mg/g) was applied to the wound (Emla, Astra Pharmaceutical, Australia) and the mice were removed from the stereotaxic 25 apparatus and monitored until fully recovered from anaesthesia. The operated mice were then returned to the animal room where they were kept for a minimum of 6 days to allow for the retrograde transport and the expression of the reporter gene.
- 30 Histology After the surgery, the mice were anaesthetised with a lethal dose of sodium pentobarbital and perfused through the heart with 0.1 M phosphate buffer saline followed by a solution of paraformaldehyde (4% in 0.1 M phosphate buffer saline). The brains were dissected out, post-fixed, embedded

- in paraffin, and cut into 10 µm-thick coronal sections. The sections were floated in a 42°C water bath and then mounted on microscope slides. The slides were heated at 60° C and subsequently immersed in a histological clearing agent (for 15 min, followed by quick rinses in graded ethanols. For 5 every injection, tissue sections at the level of the amygdala were stained with Thionin (Sigma, St Louis, MO, USA) and coverslip with DPX mounting medium (BDH Laboratory Supplies, England) to assess the location of the injections.
- 10 **Immunohistochemistry** The tissue was rehydrated in 0.1 phosphate buffer saline (PBS), placed in boiling citrate buffer (0.1 M citric acid and 0.1 M trisodium citrate, pH 6.0) for 3 min to retrieve the antigen, washed again in 0.1 M PBS, and incubated in a solution of bovine serum albumin and normal goat serum (1% and 10%, respectively in 0.1 M PBS) for 20 min. The tissue 15 was then incubated with a rabbit polyclonal antibody raised against the marker protein EGFP (anti-GFP 290, Abcam, Cambridge, UK) (dilution, 1:100 in 0.5% bovine serum albumin and 2% normal goat serum in 0.1 M PBS) for 30 min. After incubation in the primary antibody, the tissue was washed with 0.1 PBS and incubated in the dark for 30 min in Alexa Fluor 488 goat anti-rabbit 20 secondary antiserum (Molecular Probe, Eugene, OR, USA) (dilution, 1:100 in 0.5% bovine serum albumin and 2% normal goat serum in 0.1 M PBS). The tissue was subsequently washed in PBS, coverslipped with fluorescent mounting medium (DAKO, CA, USA), and kept in light-tight boxes.
- 25 **Microscopic analysis** The brain tissue was analysed to visualise the EGFP-labelled neurons with a laser scanner confocal microscope (Leica TCS SP) equipped with a krypton/argon laser. The images were acquired with excitation at 488nm and they were exported into Photoshop for presentation.
- 30 **Results**
- Figure 3 shows confocal images of the IC and the PB to illustrate the result of an injection, in the Ce, of the construct that is under the control of the NSE promoter. As predicted, such injection gave rise to EGFP-labelled neurons in

both the IC and the PB (see Figure 3). Figure 4 shows confocal images of the IC and the PB to illustrate the result of an injection, in the Ce, of the construct that is under the control of the 5 kb fragment of the GlyR gene promoter. As hypothesised, such injection gave rise to EGFP-labelled neurons in the PB
5 where GlyR is endogenously expressed but, as predicted, no EGFP-positive neurons were seen in the IC. These results show that the 5 kb fragment of the GlyR gene promoter specifically directs gene expression to a specific sub-population of neurons, i.e., GlyR-expressing neurons.

10 **Example 3 - Sequence Analysis of GlyRalpha1 promoter**

Following sequence assembly, the GLRA1 promoter contig was subjected to computational analysis. The programs Proscan v1.7, Promoter 2.0 and Promoter Inspector were used for sequence analysis of the promoter. For comparative purposes, the 1.8 kb promoter sequence from the rat NSE gene,
15 1 kb of mouse gephyrin promoter - calculated from the 'ATG', 759 bp of the GABA_AR α1 gene promoter (accession nos. S693348 and NM_00806), 1308 bp of the mouse GlyR β gene promoter (accession no. AJ300577) and 708 bp from the hGlyR α2 gene (accession no U77724) were also analysed.

20 Much of the human genome contains repetitive elements, and these retroelements can be eliminated from the computational promoter analysis using the repeat masker program. The human GlyRalpha1 sequence shown in SEQ ID No. 1 was therefore analysed to identify the presence of repetitive sequences. The results of that analysis are set out below in Tables 3 and 4.
25 The repeat masker program identified 12 retroelements within the 5723 bp GLRA1 sequence. There were also two simple and two low complexity repeats. In total they comprised 1423 bp (24.86 %) of the sequence analysed (Table 3).

30 A complete alignment of the 5723 bp GLRA1 promoter fragment, with the same sized promoter fragment from the mouse *Gra1* gene was performed using the CLUSTAL command in the MacVector program. The sequences were aligned from the translational start sites and were found to have approximately 50%

sequence identity. The alignment revealed that the larger blocks of sequence identity were between the 'ATG' translational start site and 1270 bp upstream. Alignment of the two 1270 bp fragments showed that they have 74% identity.

- 5 Due to the size restrictions of some of the programs used for identification of transcription factors, the previously published 1.1 kb GLRA1 promoter fragment was scanned for transcription factor binding sites using Sigscan, Alibaba v2.0, Match and Patch 2.3a and MatInspector professional release 5.2. Alibaba identified 93 segments as potential transcription factor binding sites,
- 10 and Transfac identified 268. AliBaba located a number of Sp1 sites, several AP-2 sites, 2 Oct-1 sites, 10 CCAAT enhancer binding protein domains, and 2 GATA-1 sequences. The TATA box was recognised as a TFIID binding site, and the CCAAT box was located at -125. There was also a cAMP response element binding site (CREB) at -395. Patch identified a number of transcription factor elements found in other neuronal genes. These included the mouse PERI 2, human NPY, rat POMC, human neu and mouse neural cell adhesion molecule (NCAM) promoter elements. Match identified 23 sites, which included a neurone-restrictive silencing factor (NRSF) that again, was completely conserved in the mouse. MatInspector identified three NRSF sites and a CREB
- 15 site, although the CREB site was in a different position to that identified by AliBaba. Elements with a 5 bp consensus are predicted to occur at least once by chance in a sequence of similar size, so were not subject to further investigation.
- 20
- 25 **Example 4 - Functional Analysis of GlyRalpha1 promoter**

Subcloning the GLRA1 Promoter

- The 5'UTR and total GLRA1 promoter sequence shown as SEQ ID No. 1 was subcloned as three promoter fragments (1.1, 3.5 and 5.7 kb). Primers 5'
- 30 GlyRP2FEco and 3' GlyRP1RevHind were designed to amplify the 1.1 kb region, excluding the 5' UTR already contained in pCis-hGlyR α 1, with the addition of restriction sites for cloning (Table 1). Human DNA extracted from blood was used as a template in the PCR. The resulting PCR fragment was digested with

- Eco*RI and *Hind*III. The hGlyR α 1 cDNA was amplified from pCis-hGlyR α 1 with 5' GlyRP5Hind and 3' GlyRP6Eco primers containing restriction sites for cloning (Table 1). The resulting 1745 bp PCR fragment, containing the entire open reading frame, 287 bp of 5'UTR and 115 bp of 3'UTR, was digested with
- 5 *Eco*RI and *Hind*III and ligated with the 793 bp *Eco*R1-*Hind*III GlyR promoter fragment. Ligation products were separated on a 0.8% 1x TAE agarose gel. Products of the correct size were excised, purified and cloned into the *Eco*R1 site in pIX.
- 10 The 3.5 and 5.7 kb promoter fragments were amplified from the cosmid with the addition of restriction enzyme sites. The 3.5 kb GLRA1 sequence was cloned into derivatives of the pIRES-EGFP vector. For the 3.5 kb pIRES-EGFP promoter construct, 3205 bp of promoter sequence was PCR amplified from the cosmid with the addition of a *Not*I site at the 5' end and a *Kpn*I site at the 3' end
- 15 (GlyRP8NotF & GlyRP10KpnR, Table 1). The hGlyR α 1 cDNA was amplified from pCis-hGlyR α 1 with primers containing *Not*I and *Kpn*I restriction sites for cloning (GlyRP7NotF & GlyRP9KpnR, Table 1). The resulting PCR fragments were digested with *Not*I and *Kpn*I and ligated together. Ligation products were separated on a 0.8% 1x TAE agarose gel. Products of the correct size were
- 20 excised, purified and cloned into the *Not*I site of a promoterless pIRES-EGFP plasmid.

In addition to the GLRA1 IRES-EGFP constructs, EGFP-fusion constructs were made with all three (1.1, 3.5, 5.7 kb) of the promoter fragments. The fusion constructs were cloned into a promoter-less pcDNA3.1 (-) plasmid (Invitrogen Corp.), where the wild type hGlyR α 1 cDNA was fused with the EGFP gene. This design was based on the N-terminal EGFP-GlyR α 1 fusion described by David-Watine *et al.*, 1999, *Neuropharmacology* 38(6): 785-92.

30 *In vitro* promoter function

A neuronal (SK-N-MC) and a non-neuronal (HEK-293) cell line were transfected with IRES-EGFP and fusion constructs containing the various GLRA1 promoter fragments. All transfections utilised the Lipofectamine 2000 reagent. Two days

- after transfection, cells were assessed for transfection efficiency. Transfection efficiency was estimated to be higher than 90% in the control transfection, and there was little cytotoxicity. However, only the 1.1 kb GLRA1 promoter was capable of driving reporter gene expression in the cell lines (data not shown).
- 5 The 1.1 kb fusion construct gave a stronger signal than the IRES-EGFP constructs – whose signal levels were too low to capture digitally, indicating that the IRES sequence is sub-optimal. As expression of the GLRA1 gene is subneuronal, i.e. largely restricted to motor neurones, the only way to test the function of the other promoter fragments is *in vivo*.

10

In Vivo Promoter Function

- As described above, a tongue injection system was designed to deliver transgene constructs directly to the hypoglossal nucleus. Injection of plasmid DNA into the tongue was followed by gene expression analysis in brainstem sections containing the hypoglossal nucleus.

- 15 The tongue injection system was used to test the function of the GLRA1 promoter fragments. Injections of IRES-EGFP plasmids carrying 1.1 kb and 3.5 kb of the hGlyRa1 promoter, both resulted in EGFP-positive motor neurones in the hypoglossal nucleus six days after injection (data not shown).

Time course of expression in hypoglossal motor neurons.

Constructs were injected into the tongue of mice as described above, except that 20 µl of DNA in 10 µg of distilled water was injected.

25

At various time points post-injection, animals were sacrificed and the brainstem dissected out, post-fixed and sectioned in 50 micron-thick coronal sections with a vibratome. The sections were mounted on microscope slides and analysed as described above.

30

The results show that the expression of EGFP protein in the hypoglossal nucleus under the control of the 5.7 kb GlyRalpha1 promoter sequence after

injection into the tongue musculature began at 4 days post injection, peaked at days 5-6 and persisted until day 14.

Discussion

5 Somatic gene delivery is a valuable tool that can be used to deliver foreign genes to the CNS. To gain access to motor neurones, a simple approach is to inject naked/pure DNA into muscle and utilise the natural retrograde processes for its transfer from the tip of the axons innervating the muscle, to the corresponding nerve cell bodies. In the present chapter, a tongue injection
10 system was used to deliver and express transgene constructs in the mouse hypoglossal nucleus. The distribution and expression of the GlyR $\alpha 1$ gene is very restricted, which makes its promoter difficult to study *in vitro*. The development of the tongue injection system made an *in vivo* analysis possible. Several GLRA1 promoter fragments of differing size were isolated and tested for
15 their ability to drive reporter gene expression.

Tongue injections of GLRA1 Promoter Fragments

As a mouse tongue is a very small piece of muscle, we had to minimise the volumes of injected DNA solution, so that physical damage and inflammation
20 would be minimal. In addition, too concentrated a DNA solution could lead to increased viscosity and difficulty in injecting the solution into the tongue. Due to these constraints, 5-10 μ g of circular plasmid DNA was injected in 8 μ l of buffer split between four sites on the animal's tongue. As injection proficiency increased, it became apparent that one midline injection of DNA in the tongue
25 was sufficient to produce the widespread reporter gene expression in the hypoglossal nucleus. In addition, the use of microinjection buffer, 1x PBS - with or without glucose, or normal saline solution as a diluent did not, at a gross level, affect expression.

30 The tongue injection system was used to show that the 1.1 kb and 3.5 kb and 5.7 kb GLRA1 promoter fragments were able to drive EGFP expression in the hypoglossal nucleus.

Neuronal Gene Regulation

Normal functioning of the nervous system relies on the neurones' ability to regulate the expression of specific neurotransmitter receptors. Expression of neural-specific genes may be pan-neuronal or sub-neuronal and have inducible qualities. However, very little is known about the DNA elements and the mechanisms that underlie this regulation.

- It has been hypothesised that neuronal gene expression can be achieved by one of four principles, which may operate alone or in combination. The first is that neurone-specific expression is conferred by a neurone-specific basal promoter.
- In this case, most of the 5' flanking region can be deleted without affecting the specificity of gene expression e.g. 255 bp of the rat NSE promoter can confer cell type specificity.
- The second principle is that a relatively non-specific/promiscuous promoter would be switched off in non-neuronal cells e.g. the rat type II sodium channel promoter contains a number of negative regulatory elements like NRSE/NRSF (neuronal restrictive silencer element/factor) that prevents expression in non-neuronal cells.
- The third *modus operandi* is that of a neurone-specific positive modulator (enhancer) that would function in neuronal cells and upregulate transcription from a constitutive minimal promoter.
- The fourth means of restricting gene expression is via a promoter that contains both a neural-specific basal promoter and upstream negative regulatory elements that restrict expression to certain subneuronal cell types.

To date, the majority of neural-specific genes that have been studied appear to use negative regulatory mechanisms to direct sub-neuronal specific expression. The presence of the three putative neural restrictive silencing factors (NRSF) elements in the GLRA1 promoter, one of which is completely conserved in the mouse GlyR α 1 promoter, suggests that this gene also uses a negative regulatory

mechanism to control its expression. This hypothesis was tested *in vitro* by transfecting a human neuronal (SK-N-MC) and a non-neuronal cell line (HEK 293) with all three of the GLRA1 promoter constructs. Promoter function, assessed by EGFP detection, revealed that only the 1.1 kb GLRA1 promoter 5 resulted in EGFP expression in the cell lines.

Early in development, GlyRs are composed of α 2 subunit homomers. They are then replaced by the GlyR α 1 and β subunits that coassemble to form the heteromeric GlyR, found in adult mice and post-natal humans. Unlike GlyR α 1, 10 the GlyR β subunit is expressed from birth and is found in areas of the brain where GlyR α 1 gene expression has not been detected. The variation in spatial and temporal expression of a gene are reflected in the promoter.

Computational analysis of the GLRA1 promoter has revealed some interesting 15 possibilities for the regulation of its gene expression. Gel shift assays could be used to examine the capacity of the DNA elements to bind transcription factors, following which, the presence of the *trans*-activating factors in the cells of interest can be confirmed. Next, the role of these putative transcription factor elements in GLRA1 regulation may be tested by deletion, rearrangement and 20 nucleotide substitution of the promoter sequence. To examine the importance of sub-neuronal specific elements, an *in vivo* system that utilises retrograde transfer of naked DNA from a discrete brain nucleus could be used, such as the tongue injection system described herein.

25 Specificity of the GLRA1 promoter

A model was developed based on a non-viral, direct brain injection method to evaluate the specificity of the GLRA1 promoter. A system based on the distinctive connectivity of the central nucleus of the amygdala was designed. 30 Figure 2 is a diagrammatic representation of the connectivity of the central nucleus of the amygdala. The central nucleus of the amygdala receives a projection from both the parabrachial nucleus, a brainstem area in which GlyR are highly expressed, and from the insular cortex where neurones do not express GlyR. It was hypothesised that an injection of the transgene under the

control of the NSE promoter, in the central nucleus of the amygdala, would result in the retrograde transport and expression of the transgene in both the insular cortex and the parabrachial nucleus. On the other hand, it was predicted that the injection of the transgene under the control of the hGlyR $\alpha 1$ gene promoter, despite being transported to both the insular cortex and the parabrachial nucleus, would only be expressed in the parabrachial nucleus. The NSE or the 5.7 kb hGlyR $\alpha 1$ promoter EGFP constructs were injected into the central nucleus of the amygdala in C57BL/6 mice. It was found that by contrast to the NSE promoter (Figure 3) the 5.7 kb hGlyR $\alpha 1$ promoter was able to drive EGFP expression in the parabrachial nucleus and not the insular cortex (Figure 4). Thus, in contrast with the 1.1 kb promoter, which has broad expression in neuronal and non-neuronal cells, the 5.7 kb promoter was clearly shown to have additional elements that restrict gene expression and confer the spatial restriction found for the endogenous GLRA1 gene.

15

Similar experiments are performed comparing the 1.1 kb promoter fragment with the 3.5 kb fragment.

In summary, the 5.7 kb hGlyR $\alpha 1$ promoter confers the natural restricted spatial expression of this gene. Whether this region also contains the regulatory elements that control the temporal aspect of GlyR $\alpha 1$ expression would need to be tested using a transgenic animal model.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are apparent to those skilled in molecular biology or related fields are intended to be within the scope of the invention.

Table 3

		Number of elements*	Length occupied	Percentage of sequence
5	SINES:	3	523 bp	9.14 %
10	ALUS	1	300 bp	5.24 %
	MIRs	2	223 bp	3.90 %
15	LINEs:	3	729 bp	12.74 %
	LINE1	0	0 bp	0.00 %
	LINE2	3	729 bp	12.74 %
	L3/CR1	0	0 bp	0.00 %
20	LTR elements:	0	0 bp	0.00 %
	MaLRs	0	0 bp	0.00 %
	ERVL	0	0 bp	0.00 %
	ERV_classI	0	0 bp	0.00 %
	ERV_classII	0	0 bp	0.00 %
25	DNA elements:	0	0 bp	0.00 %
	MER1_type	0	0 bp	0.00 %
	MER2_type	0	0 bp	0.00 %
	Total interspersed repeats:		1252 bp	21.88 %
30	Small RNA:	0	0 bp	0.00 %
	Satellites:	0	0 bp	0.00 %
	Simple repeats:	2	110 bp	1.92 %
	Low complexity:	2	61 bp	1.07 %
35	N.B. Most repeats fragmented by insertions or deletions have been counted as one element.			
	GC level:	42.46 %		

Table 4

	SW score	perc div.	perc del.	perc ins.	query sequence	begin	end	position in query (left)	matching repeat	repeat class/family	position in repeat begin end (left)	ID
	205	27.3	0.0	6.1	UnnamedSeq1	20	85	(5375)	+	L2	LINE/L2	3210 3271 (1)
	1907	13.7	3.3	0.0	UnnamedSeq1	208	507	(4953)	+	Alusq	SINE/Alu	3 312 (1)
	190	35.4	12.5	1.0	UnnamedSeq1	787	882	(4578)	+	MIR3	SINE/MIR	15 121 (87)
	180	0.0	0.0	0.0	UnnamedSeq1	1026	1045	(4415)	+	(A)n	Simple_repeat	1 20 (0)
	253	32.5	1.8	1.8	UnnamedSeq1	1427	1540	(3920)	C	L2	LINE/L2	(0) 3313 3200
	251	27.3	13.2	3.3	UnnamedSeq1	1615	1735	(3725)	+	MIR3	SINE/MIR	38 170 (38)
	252	21.7	3.3	0.0	UnnamedSeq1	1737	1796	(3664)	+	L2	LINE/L2	2598 2659 (655)
	1595	25.4	6.8	2.9	UnnamedSeq1	1829	2317	(3143)	+	L2	LINE/L2	2805 3312 (1)
	207	34.4	0.0	0.0	UnnamedSeq1	3245	3334	(2126)	+	(CATATA)n	Simple_repeat	6 95 (0)
	257	23.5	8.8	2.9	UnnamedSeq1	3452	3553	(1907)	+	MIR	SINE/MIR	152 259 (3)
	26	5.0	0.0	0.0	UnnamedSeq1	3560	3599	(1861)	+	AT rich	Low_complexity	1 40 (0)
	211	26.3	12.9	7.4	UnnamedSeq1	4079	4214	(1246)	+	MIR3	SINE/MIR	9 149 (67)
	21	0.0	0.0	0.0	UnnamedSeq1	5364	5384	(76)	+	AT rich	Low_complexity	1 21 (0)

CLAIMS

1. A polynucleotide comprising a nucleotide sequence corresponding to the 5' promoter region of a glycine receptor alpha1 subunit gene or a fragment of said sequence capable of conferring neuron-specific expression of a heterologous nucleotide sequence operably linked thereto.
2. A polynucleotide comprising a nucleotide sequence as shown in SEQ ID. No. 1, or a homologue or fragment of said sequence capable of conferring neuron-specific expression of a heterologous nucleotide sequence operably linked thereto.
3. A polynucleotide according to claim 1 or claim 2 wherein said neuron-specific expression is motor neuron-specific and/or glycine receptor expressing neuron-specific expression.
4. A polynucleotide according to claim 3 which comprises nucleotides 1 to 5397 of the nucleotide sequence as shown in SEQ ID. No. 1
5. A polynucleotide according to any one of claims 1 to 4 operably linked to a heterologous nucleic acid of interest (NOI) such that the polynucleotide directs expression of the NOI in a neuron.
6. A polynucleotide according to claim 5 wherein said neuron is a motor neuron and/or glycine receptor expressing neuron.
7. A polynucleotide according to claim 5 or claim 6 wherein the NOI encodes a polypeptide of therapeutic use.
8. A polynucleotide according to claim 5 or claim 6 wherein the NOI encodes a polypeptide which is cytotoxic.

9. A polynucleotide according to any one of claims 5 to 8 for use in delivering the NOI to a mammalian neuron.
10. A polynucleotide according to claim 9 wherein said neuron is a motor neuron and/or glycine receptor expressing neuron.
11. A nucleic acid vector comprising a polynucleotide according to any one of the preceding claims.
12. A pharmaceutical composition comprising a polynucleotide according to any one of claims 5 to 10 or a nucleic acid vector according to claim 11 together with a pharmaceutically acceptable carrier or diluent.
13. A polynucleotide according to any one of claims 5 to 10, a nucleic acid vector according to claim 11 or a pharmaceutical composition according to claim 12 for use in a method of treatment of a human or animal.
14. A polynucleotide, nucleic acid vector or pharmaceutical composition according to claim 13 wherein the polynucleotide, nucleic acid vector or pharmaceutical composition are administered to the human or animal by injection into the muscle of the human or animal such that the polynucleotide or nucleic acid vector are delivered to a neuronal cell of said human or animal by retrograde axonal transport.
15. A polynucleotide, nucleic acid vector or pharmaceutical composition according to claim 13 or claim 14 wherein the human or animal is suffering from a disease of, or injury to, the central nervous system.
16. A method of treatment of a human or animal patient suffering from a disease of, or injury to, the central nervous system, or a disease associated with the central nervous system, which method comprises administering an effective amount of a polynucleotide according to any one of claims 5 to 10, a nucleic

acid vector according to claim 11 or a pharmaceutical composition according to claim 12 to the patient in need of such treatment.

17. A method according to claim 16 wherein the polynucleotide, nucleic acid vector, viral vector or pharmaceutical composition are administered to the human or animal by injection into the muscle of the human or animal such that the polynucleotide or nucleic acid vector are delivered to a neuronal cell of said human or animal by retrograde axonal transport.

18. A method according to claim 16 or claim 17 wherein the human or animal is suffering from a disease of, or injury to, the central nervous system.

19. A method for determining whether a candidate nucleotide sequence is capable of conferring neuron-specific expression of a nucleic acid sequence of interest (NOI) operably linked thereto which method comprises:

- (i) providing a non-viral nucleotide vector comprising said candidate nucleotide sequence operably linked to an NOI;
- (ii) administering said vector to the brain or brain stem of a non-human animal; and
- (iii) determining whether said NOI is expressed specifically in neurons in the brain or brain stem of said animal.

20. A method according to claim 19 wherein said neurons in step (iii) are motor neurons and/or glycine receptor expressing neurons.

21. A nucleotide sequence capable of conferring neuron-specific expression of a nucleotide sequence of interest operably linked thereto, said sequence obtained by the method of claim 19 or claim 20.

22. A method of delivering a nucleic acid to a neuronal cell by non-viral means which method comprises administering said nucleic acid as a naked nucleic acid substantially free of complexing agents, to muscle tissue such that

the nucleic acid is transported to said neuronal cell by retrograde axonal transport.

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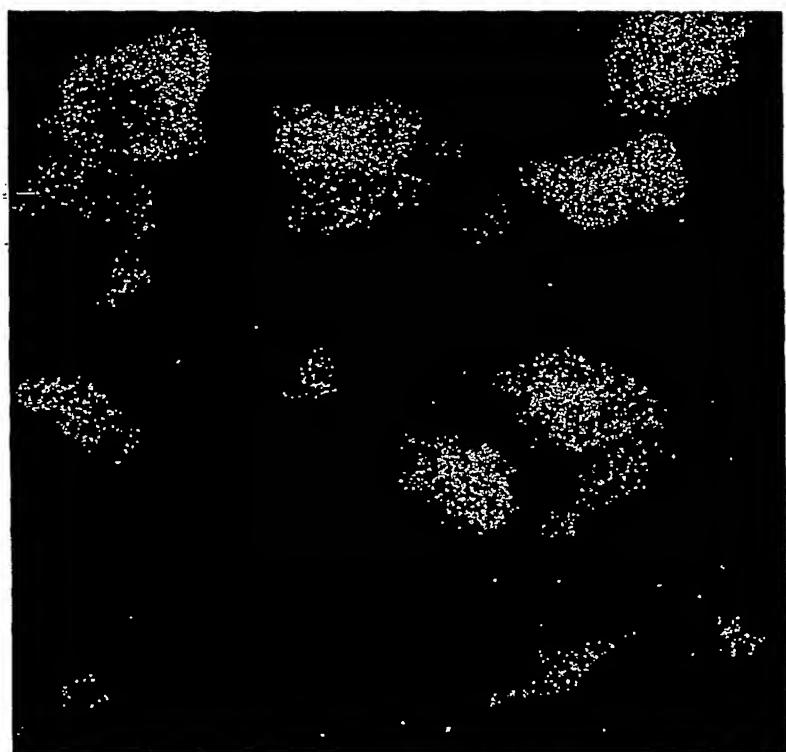
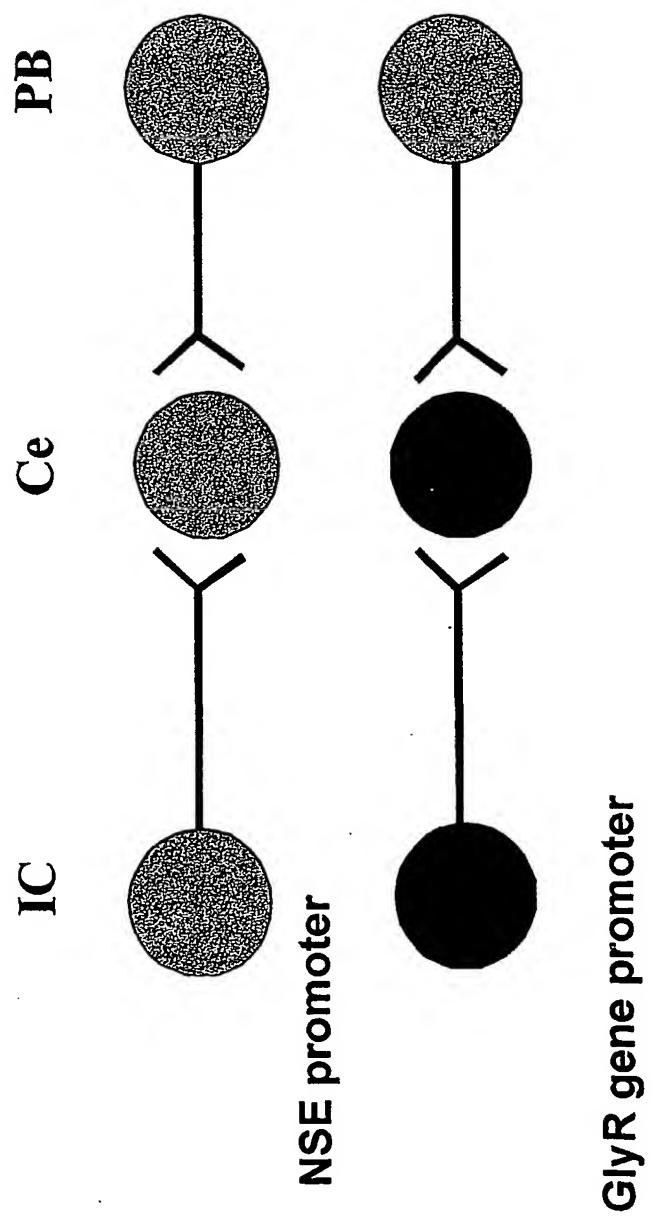


Figure 1

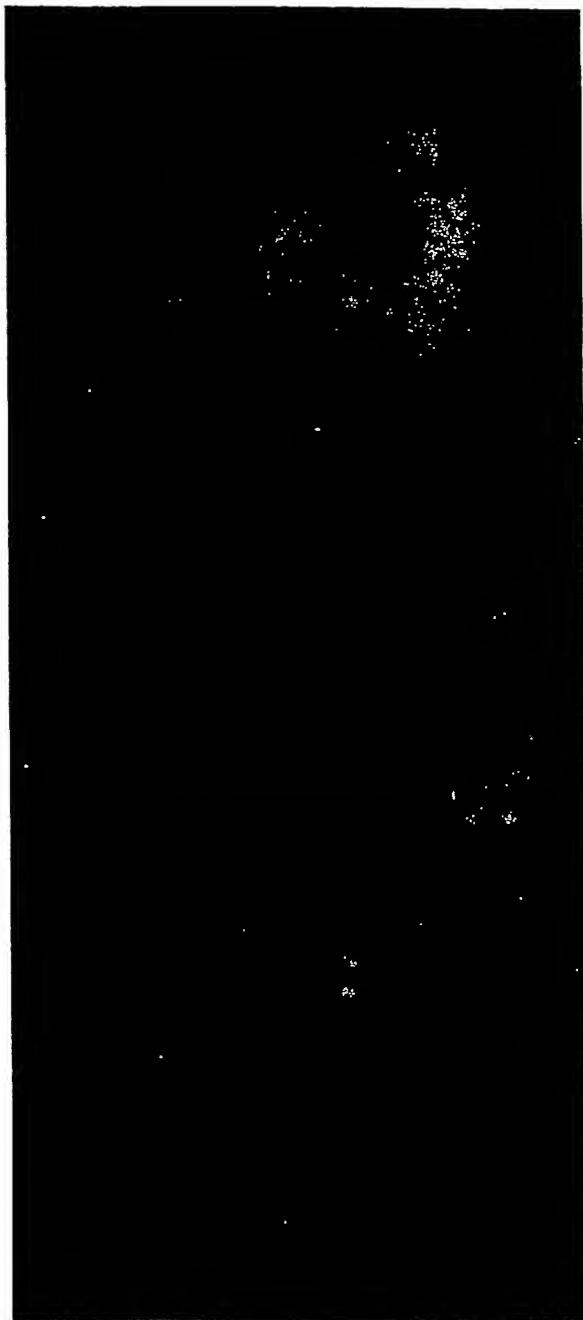
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Figure 2



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Figure 3

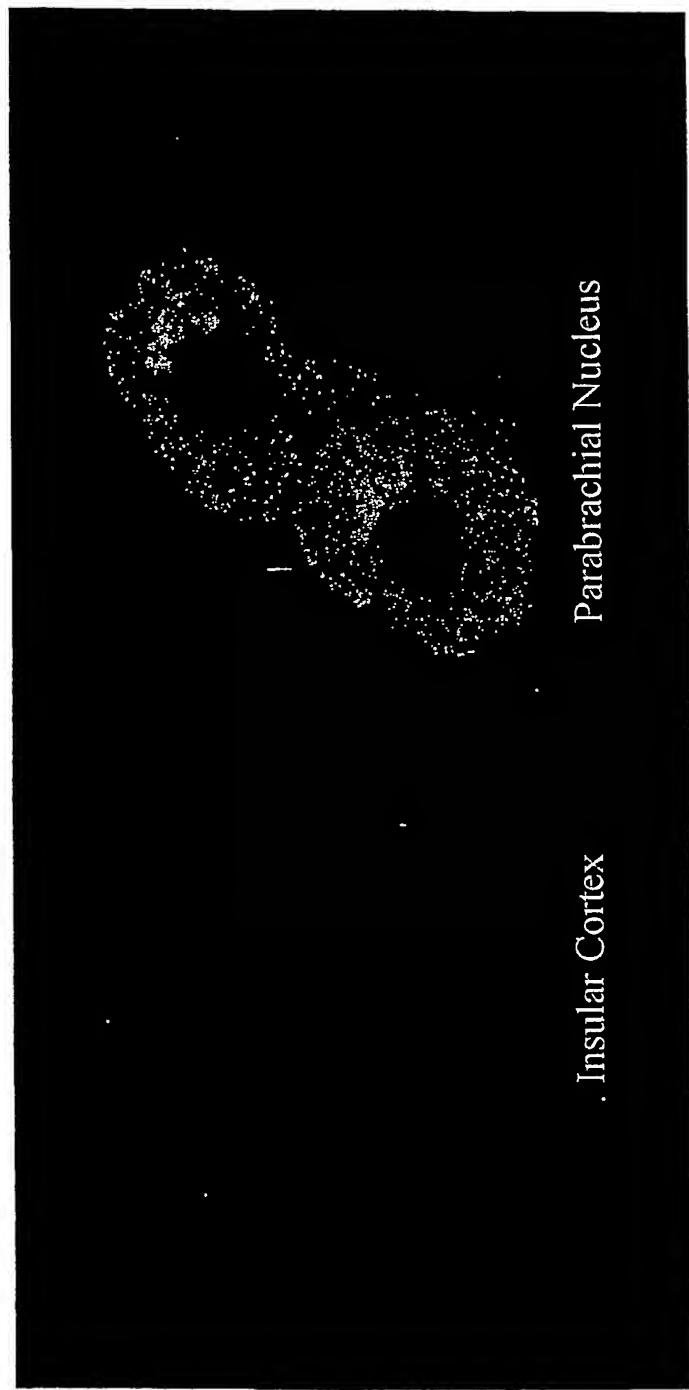


Insular Cortex

Parabrachial Nucleus

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Figure 4



SEQUENCE LISTING PART OF DESCRIPTION

SEQ ID Nos. 1, 2 - human hGlyRalpha1 promoter Sequence and cDNA
Primers were designed around the positions indicated by the bold text.

5

SEQ ID No. 1

ATCTTTATTTAGACATGTAAACCCCTCAGCACAGTGTCAAGACACACACCCAGTGTCAAGA 60
TATATATGTTTTTAATAAATGAACAAGGCTTGAGTGAATGAACAAACTCAGTAAAAG 120
10 TATATACTTATGTCAAGCCTTCATAATCCGAAAGTCTTATGGACACAGTAGGAGATGA 180
GAGATCCAATGAGAAAAACTGACTGGTCCAGACGCCGTGGCTCATGCCCTAATCCCAACA 240
CTTCGGGAGGCCAAGGCAGCGGATCACCTGGATCAGGAATTGAGACTAGCCTGGCAA 300
CATGGTGAATCCCATCTACTAAAAATACAAAATTAGCCAGGTGTGGCAGTGCACACC 360
TATAATCCCAGCTACTCGGAGGCAGGAGAACCGCTGAACCCAGTAGGTGGAGGTTGCAG 420
15 TGAGCAGAGACCACGCCACTGCACTCCAGCCTGGAAACAAGAGCAAACATCTCAA 480
AAAGAAACAGAGAGAAAGAGAAAAACTGACTGAATAAACATGAAATAACATGTAAGGA 540
CAGATGAGAAGAAAGTAGAAAAAGAGGAGAGATAGTTGAGAGAAAGATGGAAATGAG 600
GCACAGGGAGATCGCTAAAATTTCTCACAAACAGTAAGCTGTTAGAGCACCAAGTACAA 660
GTATAGACTTGGCTACAAAAGAAAATTGGGAGTCTCTGGTCTCTGAGCAGGTCACTTT 720
20 GATATGAAGCATTTCATCACAGATCTAGCTCCACTTCTGTTATTTAGGTCTCACAG 780
AAAAAAAGGGTAGGGAACCATGACCTGAAAGATAGGAGTCCTCCAACTAGATTCACTTT 840
GTTGTGGTATCTTAAGCCAGTCGTTCCCCCTTCTGGACCTTGAATAGCTAGTGAATG 900
GGAGGAAAGGTCTCTTAGTATTTATGCATCTATTCTTACAACAACTAAAATTCTGGGTCA 960
CTGAGATCCCCAAGTAGTCTGGGTCCCTTAAAGGATAAAAGAACAGGTAATTGC 1020
25 TTTGTAAAAAAAAAAAAAAAGTAACAAGAGCATTCTGGATTAGGATTAGAAGGAT 1080
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30 TGGCTCAGGACAACATAATCTCAGCTGATTAACAATATTCTTACTCTCTTCCCTGTA 1380
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TCCTTATCTTAATTGAAACAAACAAAAATAGTTGAAACACCCCTTAGCTATAATTAG 1620
35 GGTGCTTGTGTTCTGTCCCAGCTGCGCTATGAGTCTAAGGGTAAGTCTATTACCTCT 1680
CTGTATATAATTTCTCTGTACTCGAAATGTTGAACAAAGACTATCTTAAAGAATT 1740
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ATCCCAGAAATTTATTCTGGAGGTAGTACCTGCTCTAGTCCCCAACCTCCTCTAAT 1860
CTCATCTCTATCACTGTTCCCTGCACTCACTCTGTCCAGGCACACTGGTCTCCATGCTG 1920

TTTCTTGTACGCTCCAAACATACTCCCCTCTCCGGGCTTTACATTTGCTTTCCCACTG 1980
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 AATTACATAAATGCCTTTCAGGAGCCATTCTGATATGATCACCTAACATAAAATAGC 2100
 ACCTCCATGCCACTCTCTGCCCTGCCTTTTAAATTCTTATGACTGCCTAACTCA 2160
 5 TCATATATTCCTTGTGTTGCTTGTCTTCTCCAATAGAAGATGCATACTTTAGAATAG 2220
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 15 AGGCATAAAATATGTGATATGTAGGGAAAAAGTGTAGATTAACATGAGGGACTCTTCCA 2820
 CTGAACCTCTGAAGCAACTGCATGTTCCAAAAGTTATCTTATATACTGTTTATTTATA 2880
 CACTATCAGTATATACTGTTGTTATCTTACATACGTCACTATCCCCAATACTGGCA 2940
 CATGCTCATTTCATGTGTAATGATTAATGAAAGTATCAGCCTCATAGTAAACAGAGGCC 3000
 TATTAACAAACAGGTGCCCTCCACCACCCCAGGTAATAATTACTCTTACCTCCTGCACA 3060
 20 GCCCACAGCCTTGGAGCCACTCACACTCATGAACAGCAACACACACTCAGCACCCCTGA 3120
 AGGAAAAGAAAAACAAATTTCACATACGTGATCACACCTGGCTATGCAGGTATTCCTA 3180
 GTCAGATGAGGGACCAGGACAAAATTGCCAGCTACGGCTTGTTCAGTACTCTAGGAC 3240
 CACCACAAAGACACACATAGATACATACACATCTATGTGTATGTAGAGAAAAGACCCATT 3300
 ACAAAATACAAACGTACAATAACACATATGCATATCAAATCACTTCAGAATTAGGCACGA 3360
 25 TACAAATCATAAAATAATTGTATACACAGTAACCTACAAGCTGGTTTGCCTTAGCATA 3420
 CTCACAAATGTACACAAACACATACGCCAAAGATAATACTTACCTCAAGGTTGACTGTGA 3480
 AAAGTAAATGAGATTATGTGAATTACAGTAATTCTGACACATAGAAACCATGCTAATA 3540
 AAGGTTAGCTCTGATTACATATATAAAATACATATATTACATTATTTAAATTG 3600
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 TAAAAGGCAGATAAAACTGTCTCTGTCCAGTTGGAAGAAGGTGCTTTATTCCCTCTCA 4260
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CGTATTTCCCTGCTGCAGGTGACCTGGAGGGGTCTTATCCACCAGACTCTTTGG 4380
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 CAGGGATCTGGTCAACTCTCCCTTTAAAAGATAAATTGAGGCCAGAGCACGCTTA 4500
 ACAAGGAACCATGTGTTTACTCACATACAACTGCATTCTTAAAATCAAGGAAACCTT 4560
 5 TTGAGAGACCTAGAGAGATTAATTCCATATCCTCTCACCCACCGCTTACACGAAAG 4620
 AAGCTTGGGGTAGGAAGGGATTGCGCCAGATCTCGTCCAGCACTCTAACGTTAAATT 4680
 CAACCCGGAGTCTAACAGGCTATTGGCGAATACCGGGAGAAGATCTGGTTTCCGTAGC 4740
 CGCGGGCCCAATGAACACCTCGAGTGGGAGGGTTGAAGATACTAAGGAGTTTCCCTCA 4800
 ACCCCCACCCCCATCGGTATCACCCCTCCCATCCCCCACGCCCTCCAGTTGTTAAATT 4860
 10 AATGCAGTAAGAAAATCTGAAGATCTGAAGGAGTCTTGACCAAGGAAAGATGAGCGAGCT 4920
 CAGAAAACGAGATGCATCTGAGACATTGGCTTCAGAGCCCCGGTAGCCAGGGACCCGG 4980
 TAGCCCCCTGAAACCTGCCCCCTGGGCTCTCTGACTGGCGCCCCGAGCAATGGACACCTT 5040
 TCTGCAGTGATCACAACTTATTGGCGGGACCAGGAAACAGGAATAGCAAGGCGACAGCA 5100
 GCCGGACAGAGGCAGGAGAACGAGATTGGATTGCGATGCCAGCCTGGCAC 5160
 15 ACTCTCCGAGATTTAATCTGAAGGCAGCGCCGTCAACCGCTTCTCCCTGCCCTAAACCC 5220
 CTCAGGCCGGCCAGAGCCATAAAGCTCTGTTCTTACCAAGATGCTGCCCAATCC 5280
 AACGGTCCGAGGCCAGCGCTCTGATCTCCACAGAAGATTTTCTTGAGGGAGCTGG 5340
 CCGTTTAAACAGAAAACAGGGTTAAAAAAAAAAAAAAAAAGGAAATTACCCACCC 5400
 CCAAACGTGCCTCCCCAGGCCGCAGGGAGCCAACAGACACGCTGGAGTTAACAAACAG 5460
 20 CAATACTCTCGCGCTCCTGAAAAGCAGGTCTGGACGCTCTCCGTGGTGTGAAACGCCT 5520
 CGCAGCCGCCGCTGTCCGTGGTATCTACGACCCCTCGCTCCAATTCCCTGGGCTCT 5580
 CCCTCCGCCCTGGTCCCCGCCCTTAAACATCTGGATTATTGGCAATAGCGC 5640
 TTTCTGGTTTGTAAAGTCCAATTGAAACATTGGCCCTAACTCGTGGACTACA 5700
 AAGCACAAAGGACCTGAAAA 5720

25

SEQ ID No. 2**5' UTR and cDNA from hGlyRalpha1 in the pCis vector**

30 AGGGAGCCAACAGACACGGCTGGAGTTAACAAAAGCAATACTCTTCGGCTCCTGAAAAGCAGGT 66
 CTGGACGCTCCCGTGGTGTAAACGCCCTCGCAGCCGCCGCTGTCCGTGGTATCTACGACCCCT 132
 CGCTCCAATTCCCTGGGCTCTCCCTCCGCCCTGGTCCCCGCCCTTAAACATCTGGAT 198
 TATTTTTTGCAATAGCGTTCTGGTTTGTAAGTCCAATTGAAACATTGGCCCTAAAC 264
 TCGTGGACTACAAGCACAAAGGACCTGAAAATGTACAGCTCAATACTCTTCGACTCTACCTT 330
 M Y S F N T L R L Y L

GGGAGACCATTGTATTCTCAGCCTGCTGCTCTAAGGAGGCTGAAGCTGCTCGCTCCGCACCCA 396
 W E T I V F F S L A A S K E A E A A R S A P

40 AGCCATATGTCAACCTCGGATTCCTGGATAAGCTAATGGGAGAACCTCCGGATATGATGCCAGGA 462
 K P M S P S D F L D K L M G R T S G Y D A R

TCAGGCCAATTAAAGGTCCCCAGTGAACGTGAGCTGCAACATTTCATCAACAGCTTGGTT 528
 I R P N F K G P P V N V S C N I F I N S F G

45

CCATTGCTGAGACAACCATGGACTATAGGGTCAACATCTCCTGCGCAGCAATGGAACGACCCCC 594
 S I A E T T M D Y R V N I F L R Q Q W N D P

 5 GCCTGGCCTATAATGAATAACCTGACGACTCTCTGGACCTGGACCCATCCATGCTGGACTCCATCT 660
 R L A Y N E Y P D D S L D L D P S M L D S I

 GGAAACCTGACCTGTTCTTGCCAACGAGAAGGGGGCCCACCTCCATGAGATCACCACAGACAACA 726
 W K P D L F F A N E K G A H F H E I T T D N

 10 AATTGCTAAGGATCTCCCGGAATGGAATGTCCTCTACAGCATCAGAACATCACCTGACACTGGCCT 792
 K L L R I S R N G N V L Y S I R I T L T L A

 GCCCCATGGACTTGAAGAATTCCCCATGGATGTCCAGACATGTATCATGCAACTCGAGAGCTTG 858
 C P M D L K N F P M D V Q T C I M Q L E S F

 15 GTTATACGATGAATGACCTCATCTTGAGTGGCAGGAACAGGGAGCCGTGCAGGTAGCAGATGGAC 924
 G Y T M N D L I F E W Q E Q G A V Q V A D G

 20 TAACTCTGCCCAAGTTATCTTGAGGAAGAGAAGGACTTGAGATACTGCACCAAGCACTACAACA 990
 L T L P Q F I L K E E K D L R Y C T K H Y N

 CAGGTAATTACCTGCATTGAGGCCGATTCCATCTAGAGCGGCAGATGGCTACTACCTGATTC 1056
 T G K F T C I E A R F H L E R Q M G Y Y L I

 25 AGATGTATATTCCCAGCCTGCTCATGTCATCCTCTCATGGATCTCCTCTGGATCAACATGGATG 1122
 Q M Y I P S L L I V I L S W I S F W I N M D

 CTGCACCTGCTCGTGTGGCCTAGGCATCACCCTGTGCTCACCATGACCACCCAGAGCTCTGGCT 1188
 A A P A R V G L G I T T V L T M T T Q S S G

 30 CCCGAGCCTCCCTACCCAAGGTGTCTACGTGAAAGCTATTGACATCTGGATGGCTGTTGCCGTGC 1254
 S R A S L P K V S Y V K A I D I W M A V C L

 35 TCTTCGTGTTCTGGCCCTGCTGGAATATGCCGCTGTCAACTTGTGCTCGCAACATAAGGAGC 1320
 L F V F S A L L E Y A A V N F V S R Q H K E

 TGCTCCGATTCAAGGAGGAAGCGGAGACATCACAGGAGGATGAAGCTGGAGAAGGCCGCTTAACT 1386
 L L R F R R K R R H H K E D E A G E G R F N

 40 TCTCTGCCTATGGGATGGGCCAGCCTGTCTACAGGCCAAGGGATGGCATCTCAGTCAAGGGGCCA 1452
 F S A Y G M G P A C L Q A K D G I S V K G A

 ACAACAGTAACACCACCAACCCCCCTCCTGCACCATCTAAGTCCCCAGAGGAGATGGCAAAACTCT 1518
 N N S N T T N P P P A P S K S P E E M R K L

 45 TCATCCAGAGGGCCAAGAAGATCGACAAATATCCCGATTGGCTTCCCCTGGCTTCCATGGCTTCCATTT 1584
 F I Q R A K K I D K I S R I G F P M A F L I

 50 TCAACATGTTCTACTGGATCATCTACAAGATTGTCCGTAGAGAGGACGTCCACAACCAAGTGAAGGG 1650
 F N M F Y W I I Y K I V R R E D V H N Q

 TCTGAAAGGTTGGGGAGGGCTGGAGAGGGAACGTGGAAATGACAGGAATCTGAGAGACGGTC 1716
 GACTAGAGTCGACCTGCAGCCCAAGCTAAC

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU02/00711

A. CLASSIFICATION OF SUBJECT MATTER																						
Int. Cl. ⁷ : C12N15/11, C12P 21/02, C07K14/48.																						
According to International Patent Classification (IPC) or to both national classification and IPC																						
B. FIELDS SEARCHED																						
Minimum documentation searched (classification system followed by classification symbols) World Patent Index (WP) Chemical Abstracts (CA). Key words (KW): see electronic data base box below. Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Medline(MD), Genbank PIR Swiss-Prot, EMBL. Key words (KW): see electronic data base box below. Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) KW used in WP, CA, MD: Promoter and glycine receptor, GenBank, EMBL, Swiss-Prot and PIR sequence id no 1.																						
C. DOCUMENTS CONSIDERED TO BE RELEVANT																						
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.																				
X	EMBL accession no U77732 "Homo sapiens glycine receptor alpha 1 subunit gene, partial cds." Published 15 December 1996. The disclosed sequence has 99.9 % identity to seq id no 1 of the application (at position 4621 to 5720 of the latter).	1-4 and 11.																				
X	Monani U and Burghes AHM "Structure of the human α_2 subunit gene of the glycine receptor -- use of vectorette and Alu-Exon PCR." Genome Research 6:1200-1206 (1996) See the whole document especially figure 1 B and pages 1203 (2 nd column) to page 1205.	1-11 and 19-21.																				
X	Grenningloh G et al "Alpha subunit variants of the human glycine receptor: primary structures, functional expression and chromosomal localization of the corresponding genes." The EMBO Journal vol 9 no 3 pp 771-776, 1990. See the whole document, especially page 774 (2 nd column) and figure 1B.	1-11 and 19-21.																				
<input type="checkbox"/> Further documents are listed in the continuation of Box C		<input type="checkbox"/> See patent family annex																				
<p>* Special categories of cited documents:</p> <table> <tr> <td>"A"</td> <td>document defining the general state of the art which is not considered to be of particular relevance</td> <td>"T"</td> <td>later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>"B"</td> <td>earlier application or patent but published on or after the international filing date</td> <td>"X"</td> <td>document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>"L"</td> <td>document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>"Y"</td> <td>document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"O"</td> <td>document referring to an oral disclosure, use, exhibition or other means</td> <td>"&"</td> <td>document member of the same patent family</td> </tr> <tr> <td>"P"</td> <td>document published prior to the international filing date but later than the priority date claimed</td> <td></td> <td></td> </tr> </table>			"A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"B"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family	"P"	document published prior to the international filing date but later than the priority date claimed		
"A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention																			
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"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art																			
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family																			
"P"	document published prior to the international filing date but later than the priority date claimed																					
Date of the actual completion of the international search 12 August 2002	Date of mailing of the international search report 21 AUG 2002																					
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer J.H. CHAN Telephone No : (02) 6283 2340																					

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU02/00711

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Matzenbach B <i>et al</i> "Structural analysis of mouse glycine receptor α subunit genes. Identification and chromosomal localization of a novel variant, $\alpha 4$." The Journal of Biological Chemistry vol 269 no 4 issue of January 28, pp 2607-2612, 1994.	
A	Ramming M <i>et al</i> "Analysis of the promoter region of the murine gephyrin gene." FEBS Letters 405 (1997) 137-140.	